An evolutionary perspective on (chronic) disease

Ruiz Nunez, Begona

AN EVOLUTIONARY PERSPECTIVE ON (CHRONIC) DISEASE:

lifestyle, nutritional imbalances and low-grade inflammation

Begoña Ruiz-Núñez

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CHAPTER 1.1

Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low grade inflammation in an evolutionary context

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Abstract

In this review, we focus on lifestyle changes, especially dietary habits, that are at the basis of chronic systemic low grade inflammation, insulin resistance and Western diseases. Our sensitivity to develop insulin resistance traces back to our rapid brain growth in the past 2.5 million years. An inflammatory reaction jeopardices the high glucose needs of our brain, causing various adaptations, including insulin resistance, functional reallocation of energy-rich nutrients and changing serum lipoprotein composition. The latter aims at redistribution of lipids, modulation of the immune reaction, and active inhibition of reverse cholesterol transport for damage repair. With the advent of the agricultural and industrial revolutions, we have introduced numerous false inflammatory triggers in our lifestyle, driving us to a state of chronic systemic low grade inflammation that eventually leads to typically Western diseases via an evolutionary conserved interaction between our immune system and metabolism. The underlying triggers are an abnormal dietary composition and microbial flora, insufficient physical activity and sleep, chronic stress and environmental pollution. The disturbance of our inflammatory/anti-inflammatory balance is illustrated by dietary fatty acids and antioxidants. The current decrease in years without chronic disease is rather due to 'nurture' than 'nature', since less than 5% of the typically Western diseases are primary attributable to genetic factors. Resolution of the conflict between environment and our ancient genome might be the only effective manner for 'healthy aging', and to achieve this we might have to return to the lifestyle of the Paleolithic era as translated to the 21st century culture.

Keywords

Chronic systemic low grade inflammation, evolution, brain, encephalization quotient, immune system, diet, fatty acids, fish oil, fruits, vegetables, antioxidant network, metabolic syndrome, glucose, homeostasis, insulin resistance, cholesterol, lifestyle, antioxidants, resoleomics, pro-inflammatory nutrients, anti-inflammatory nutrients.

List of abbreviations

AA, arachidonic acid; ADHD, attention deficit hyperactivity disorder; AHA, American Heart Association; ALA, alpha-linolenic acid; BMI, body mass index; CARS, compensatory anti-inflammatory response syndrome; CAT, catalase; COX-2, cyclo-oxygenase-2; CRP, C-reactive protein; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EBM, evidence based medicine; EBN, evidence based nutrition, EPA, eicosapentaenoic acid; EQ, encephalization quotient; FADS2, Δ-6-desaturase; FDB, familial defective apo-B100; FH, familial hypercholesterolemia; GPR120, G-protein-coupled receptor 120; GPx, glutathione peroxidase; GWAS, genome wide association studies; HDL, high density lipoprotein; HPA-axis, hypothalamus-pituitary-adrenal gland axis; HPG-axis, hypothalamus-pituitary-gonadal gland axis; HPL, human placental lactogen; HPT-axis, hypothalamic-pituitary-thyroid axis; IGF-1, insulin-like-growth factor-1; LA, linoleic acid; LCP, long-chain polyunsaturated fatty acids; LDL, low density lipoprotein; LOX-12, lipoxygenase-12; LOX-15, lipoxygenase-15; LOX-5, lipoxygenase-5; LPS, lipopolysaccharides; LTB_4, leukotrienes-B_4; LX, lipoxin; NFκB, nuclear factor kappa B; NTIS, non-thyroidal illness syndrome; PGD_2, prostagladins- D_2; PGE_2, prostagladins-E_2; PLA_2, phospholipase A_2; PPAR; peroxisome proliferator activated receptor; RCT, randomized controlled trial; ROS, reactive oxygen species; RR, relative risk; SAA, serum amyloid A; SIRS, systemic inflammatory response syndrome; TNFα, tumor necrosis factor alpha; TSH, thyroid stimulating hormone; VLDL, very low density lipoprotein.

Introduction

In recent years, it has become clear that chronic systemic low grade inflammation is at the basis of many, if not all, typically Western diseases centered on the metabolic syndrome. The latter is the combination of an excessive body weight, impaired glucose homeostasis, hypertension and atherogenic dyslipidemia (the 'deadly quartet'), that constitutes a risk for diabetes mellitus type 2, cardiovascular disease (CVD), certain cancers (breast, colorectal, pancreas), neurodegenerative diseases (e.g. Alzheimer's disease), pregnancy complications (gestational diabetes, preeclampsia), fertility problems (polycystic ovarian syndrome) and other diseases [1]. Systemic inflammation causes insulin resistance and a compensatory hyperinsulinemia that strives to keep glucose homeostasis in balance. Our glucose homeostasis ranks high in the hierarchy of energy equilibrium, but becomes ultimately compromised under continuous inflammatory conditions via glucotoxicity, lipotoxicity, or both, leading to the development of beta-cell dysfunction and eventually type 2 diabetes mellitus [2].

Insulin resistance has a bad name. The ultimate aim of this survival strategy is, however, deeply anchored in our evolution, during which our brain has grown tremendously. The goal of reduced insulin sensitivity is, among others, the reallocation of energy-rich nutrients because of an activated immune system [3, 4], limitation of the immune response, and the repair of the inflicted damage. To that end, serum lipoproteins adopt a pattern that bears resemblance with the 'hyperlipidemia of sepsis', accompanied by seemingly inconsistent changes in serum cholesterol, increased triglycerides, decreased HDL-cholesterol, and an increase of 'small dense' LDL-particles, of which the latter three constitute the triad of atherogenic dyslipidemia that is part of the metabolic syndrome [5–10].

From the perspective of our brain growth during evolution, we address the question of why *homo sapiens* is so sensitive to the development of insulin resistance. The purpose and the underlying mechanisms leading to insulin resistance and the associated dyslipidemia are subsequently discussed in more detail. We argue that our current Western lifestyle is the cause of many false inflammatory triggers which successively lead to a state of chronic systemic low grade inflammation, insulin resistance, the metabolic syndrome, and eventually to the development of the above mentioned typically Western diseases of affluence. To find a solution for the underlying conflict between our environment and our ancient genome, we also go back in time. With the reconstruction of our Paleolithic diet, we might be able to obtain information on the nutritional balance that was at the basis of our genome. We argue that insight into this balance bears greater potential for healthy aging than the information from the currently reigning paradigm of 'Evidence Based Medicine' (EBM) and 'randomized controlled trials' (RCTs) with single nutrients.

Our brain growth rendered us sensitive to glucose deficits

Homo sapiens and the current chimpanzees and bonobos share a common ancestor, who lived in Africa around 6 million years ago. Since about 2.5 million years ago, our brain has strongly grown from an estimated volume of 400 mL to the current volume of approximately 1,400 mL (Figure 1). This growth was enabled by the finding of a high-quality dietary source, that was easy to digest and contained an ample amount of nutrients, necessary for the building and maintenance of a larger brain. The nutritional quality of primate food correlates positively with *relative* brain size and inversely with body weight, suggesting that a larger brain requires a higher dietary quality [11]. The necessary so-called 'brain selective nutrients' include, among others, iodine, selenium, iron, vitamins A and D, and the fish oil fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that jointly are abundantly available in the land-water ecosystem. There are compelling arguments that a sizeable part of our evolution occurred at places where the land meets the water [12–15], but also that we have changed our lifestyle in a too short period of time. These changes started from the agricultural revolution (around 10,000 years ago) and became accelerated since the industrial revolution (about 100–200 years ago). They created a conflict between our current lifestyle, including our diet, and our ancient genome, that, with an average effective mutation rate of 0.5% per million years, still resides for the greater part in the Paleolithic era [16, 17]. It is not by chance that the above-mentioned brain selective nutrients are among those of which we currently exhibit the largest deficits worldwide. These deficits are masked by enrichment and fortification of our current diet with iodine (in salt), vitamins A and D (e.g. in margarines and milk) and iron (flour, cereals).

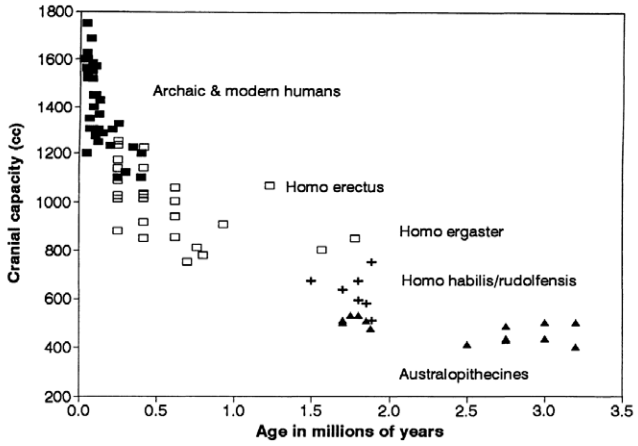

Figure 1. Evolution of our brain size within the past 3.5 million years.
Our brain has grown fast since the *homo erectus* (1.7-2.0 million years ago). The newborn *homo sapiens*, the adult chimpanzee and the *homo floresiensis* [18] have brain volumes of around 400 mL. Adapted from Aiello and Wheeler [19] with permission from The University of Chicago Press.

Our brain consumes 20–25%[1] of our basal metabolism [11–17, 20] and is thereby together with the liver (19%[2]), our gastrointestinal tract (15%[2]), and skeletal musculature (15%[2]) among the quantitatively most important organs in energy consumption [19]. The infant brain consumes as much as 74% of the basal metabolism [11, 21]. In contrast to most other organs, the brain uses mostly glucose as an energy source. There is no other primate equipped with such a large, glucose-consuming, luxury organ as our brain. For example, our closest relative, the chimpanzee, has a brain volume of 400 mL, which consumes about 8–9% of the basal metabolism. Because of the high energy expenditure of a large brain, it was necessary to make various adjustments in the sizes of some other organs. There is a linear relationship between body weight and basal metabolism among terrestrial mammals (Figure 2). This apparently dogmatic relationship predicts that, due to the growth of our brain, other organs with high energy consumption had to be reduced in size, what in evolution is known as a 'trade-off'[2]. As a consequence of this 'expensive tissue hypothesis' of Aiello and Wheeler [19] our intestines, amongst others, had to become reduced in size. However, this exchange of expensive tissue probably occurred prior to, or simultaneous with, our brain growth, in which the trigger was the consumption of the easily digestible high-quality food [20] that contains the above-mentioned 'brain selective nutrients' from the land-water ecosystem. Under these 'conditions of existence' (Darwin), a single mutation in a growth regulatory gene is likely to have been sufficient for the brain to grow. This notion derives from the existence of genetically-determined micro- [22] and macrocephaly [23] and it is as a 'proof of principle' demonstrated by the differences in the beak lengths of Darwins' legendary Galapagos finches [24–26]. Compared with our close (vegetarian) relatives in the primate world, we possess a relatively long small intestine and a relatively short

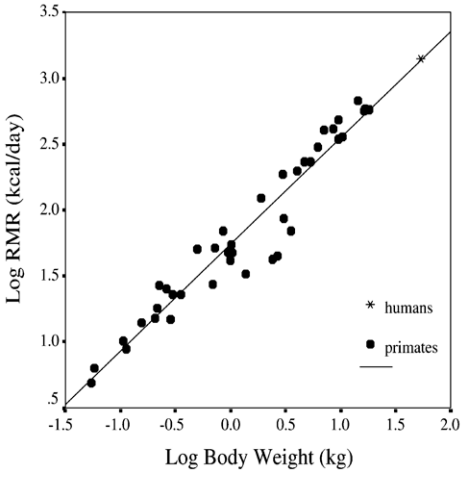

Figure 2. Relationship between body weight and basal metabolism in 51 land mammals (20 non-primates, 30 primates, and humans).
Adapted from Leonard et al.[11] with permission from Elsevier.

1 These estimates derive from various publications and therefore do not add to 100%. They should be regarded as indications.

2 The beneficial exchange of a certain property into another one

large intestine, which corresponds with the digestion of high quality food (such as meat and fish) in the small intestine, and the lesser need of a long colon for the digestion of complex carbohydrates (e.g. fiber) from a typically vegetarian diet [19]. Unlike our near primates, such as the gorilla, our teeth and the attachments of our jaw muscles are not specialized for the processing of tough vegetarian food. Also our muscle mass became adapted, since its current size is relatively small compared to our body weight. For instance, when compared with the chimpanzee, we are definitely weak. On the other hand, we have a relatively sizeable fat mass, which probably serves as a guarantee for the high energy requirement of our brain.

Our brain's energy consumption is quite stable. Unlike other organs, the energy consumption of the brain can not be downregulated at times of a negative energy balance or fasting [11, 20]. Our brain also gets spared during prolonged fasting, while other organs such as the liver, spleen, kidneys and even the heart, are sacrificed for energy generation [27]. This hierarchy also applies to the prenatal brain, whose development is conserved during intrauterine growth restriction [28]. An example is the Indian 'thin fat baby', with a birth weight of 2,700 g. Compared with its 3,500 g counterpart from the UK, this infant has a similar brain size and a relatively large fat compartment, at the expense of the somatic growth of the skeletal muscle, kidneys, liver and the pancreas [28]. Our brain ranks high in the functional hierarchy and should be provided with the necessary energy at all times.

Apart from its large size, there is nothing special about our brain within the primate world. Compared with other species, primates have a very economical space-saving brain, but among the primates, brain weight correlates with the number of neurons [29–32] and intelligence [33]. Actually, our brain is no more than an oversized primate brain [29]. What does distinguish us from other species is the high ratio between our brain size and our body weight, which is also named encephalization quotient (EQ) (Figure 3). Toothed whales (brain weight 9,000 g) and African elephants (4,200 g) have much larger brains than humans, but they have lower EQs [34]. Among the primates, EQ does not correlate with intelligence [33]. Our high EQ has major implications for our energy management, particularly at times of 'glucose shortage'. Under normal circumstances, our brain functions almost entirely on glucose, consuming up to 130 g/day [27]. Compared with the apparently unlimited storage capacity for fat, we only dispose of a small reserve of glucose that is stored as glycogen in the liver (up to 100–120 g; mobilizable) and muscles (360 g; for local usage), while some glycogen can even be found in brain's astrocytes [35]. With the exception of the glycerol moiety, we can not convert fat into glucose. The reduced carbohydrate intake that came along during evolution with the transition from vegetarians to omnivores rendered us strongly dependent on gluconeogenesis from (glucogenic) amino acids. This was possible because we simultaneously consumed more protein from meat and fish, which is also referred to as the 'carnivore connection' [36]. After the depletion of our glycogen reserves, for instance after an overnight fast, we obtain the necessary glucose for our brain via gluconeogenesis from glycerol and amino acids. Under normal conditions, these amino acids derive from our dietary proteins after a meal, but during starvation, they become extracted from our tissues by catabolism of functional proteins, at the expense of our lean body mass. Under such circumstances of severe glucose deficit, the energetic need of our brain becomes increasingly covered by ketone bodies from fat [37, 38].

A glucose deficit leads to competition between organs for the available glucose. As previously mentioned, this occurs during fasting, but also during pregnancy and infection/inflammation. Fasting is characterized by a generalized shortage of glucose (and other macronutrients), but in case of pregnancy and inflammation we deal with active compartments competing with the brain for the available glucose, i.e. the growing child and the activated immune system, respectively. During competition between organs for glucose, we fulfill the high glucose needs of the brain by a reallocation of the energy-rich nutrients, and to that end, we need to become insulin resistant.

Reallocation of energy-rich nutrients by insulin resistance

The developing child grows fast in the third trimester of pregnancy. In this period, the supply of the necessary building blocks like glucose and fatty acids should be independent of the maternal metabolic status, which is known as the state of 'accelerated starvation' and 'facilitated anabolism' [38]. Glucose crosses the placenta without restriction. Fetal needs are directive, since the developing fetus is high in the evolutionary hierarchy. If necessary, the fetal needs become covered at the expense of the mother, which is known as the 'depletion syndrome'.

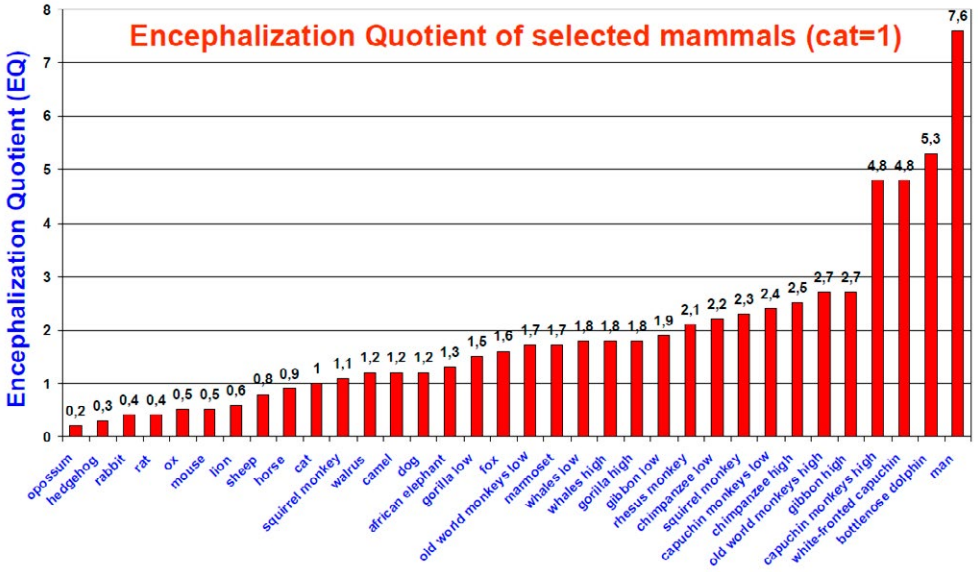

Figure 3. Encephalization quotient (EQ) of selected mammals.
The EQ has been normalized with the cat as a reference. Data adapted from Roth and Dicke [34].

During infection/inflammation we deal with the metabolic needs of an activated immune system for acute survival. The inactive immune system consumes about 23%[2] of our basal metabolism, of which as much as 69% derives from glucose (47%) and the glycogenic amino acid glutamine (22%). Upon activation, the energy requirement of our immune system may increase with about 9–30% of our basal metabolic rate. In multiple fractures, sepsis and extensive burns, we deal with increases up to 15–30, 50, and 100% of our basal metabolism, respectively [3, 4, 39].

The way we save glucose for our brain during starvation, for the brain and the fetus during pregnancy, and for the brain and immune system during infection/inflammation, is by causing insulin resistance in selected insulin-dependent tissues. These tissues are thereby forced to switch to the burning of fat. Due to insulin resistance, the adipose tissue compartment will be encouraged to distribute free fatty acids, while the liver will be encouraged to produce glucose via gluconeogenesis and to distribute triglycerides via VLDL. The aforementioned (asymmetric) 'thin fat baby' with its spared brain, relatively high adipose tissue compartment, and the growth restricted body (islets of Langerhans included), has relatively high cord plasma insulin and glucose concentrations at birth [28]. These characteristics of insulin resistance and diabetes mellitus are probably necessary for the postpartum, saving of as much as possible of the available glucose for the brain, whereas the other organs are provided with fatty acids from the sizeable adipose tissue stores. This intrauterine 'programming', that follows the prediction of a thrifty postnatal life comes along with health risks, notably when the prediction proves false [40, 41]. According to the 'Barker hypothesis', at adult age, these children have a higher chance of diseases related to the metabolic syndrome, especially when they are raised in our current obesogenic society. The unfavorable interaction of their high EQ with a high body weight is already demonstrable at the age of 8 years [42]. Essentially, their postnatal risk is attributable to a (probably epigenetic) 'intrauterine programming', that traces back to the high hierarchical ranking of our brain in both growth and energy needs, also referred to as 'the selfish brain' [43].

Glucose intolerance [26] and insulin resistance have been reported in calorie restriction, extremefasting and anorexia nervosa, and may even cause, under these circumstances, diabetes mellitus type 2, notably in those subjects sensitive to its development [44]. According to textbooks, insulin resistance during the third trimester of pregnancy is caused by the hormonal environment, among which HPL, progesterone,

estrogens, prolactin and cortisol are mentioned. However, placental tumor necrosis factor alpha (TNFα) correlates best with measures of maternal insulin resistance [45, 46]. Pregnancy is therefore sometimes referred to as a physiological state of systemic low grade inflammation [47]. As a consequence of reduced insulin sensitivity, maternal circulating concentrations of energy-rich nutrients, such as glucose and fat, tend to increase, promoting their transport across the placenta. Under non-pregnant conditions, this situation would resemble pathology, but is tolerable during the 9 months of a pregnancy, while the largest changes occur during the third trimester.

During infection and inflammation, the signals for metabolic adaptation become transmitted by pro-inflammatory cytokines. The resulting insulin resistance causes reallocation of energy (i.e. the aim of the process; see above), which illustrates that inflammation and metabolism are highly integrated [49-51]. At the molecular level, the interaction takes place through the influences of the nuclear factor kappa B (NFκB) and the AP-1 Fos/June inflammatory pathways on the PI3K/Akt signal transduction pathway for nutrient metabolism and the Ras/MAPK pathway for gene expression, which are both part of the insulin signal transduction [48, 52]. To put it simply: the activated inflammatory signal transduction pathway causes inhibition of the postreceptor insulin signaling pathway, which becomes noticeable by what we know as insulin resistance (Figure 4). Insulin resistance especially refers to a grossly diminished reduction of the circulating glucose concentration by insulin. However, insulin has many functions, and thereby exerts different effects in the various organs carrying the insulin receptor. Consequently, the 'resistance' affects the many insulin signal transduction pathways at various degrees, and thereby works out differently with respect to the various insulin functions [1, 53]. Some processes are impaired (i.e. are genuinely 'resistant'), while others remain intact and become excessively stimulated by the compensatory hyperinsulinemia. This compensatory increase of the circulating insulin levels aims at the prevention of a disturbed glucose homeostasis and thereby the onset of type 2 diabetes mellitus. The persistence of compensatory hyperinsulinism is responsible for most, if not all, of the abnormalities that belong to the metabolic syndrome [1].

In muscle and fat cells, insulin resistance induces a diminished glucose uptake and therefore a reduced storage of glucose as glycogen and triglycerides. In fat cells, it causes decreased uptake of circulating lipids, increased hydrolysis of stored triglycerides and their mobilization as free fatty acids and glycerol. In liver cells, insulin resistance induces the inability to suppress glucose production and secretion, in addition to decreased glycogen synthesis and storage. The hereby promoted reallocation of energy-rich substrates (glucose to the brain, fetus and immune system; fat to the fetus and the organs that became insulin resistant) and the compensatory hyperinsulinemia, are meant for short-term survival, and their persistence as a chronic state are at the basis of the ultimate changes that we recognize as the symptoms of the metabolic syndrome, including the changes in glucose and lipid homeostasis [3, 4] and the increasing blood pressure. For example, the concomitant hypertension has been explained by a disbalance between the effects of insulin on renal sodium reabsorption and NO-mediated vasodilatation, in which the latter effect, but not the first, becomes compromised by insulin resistance, causing salt sensitivity and hypertension [54].

Reaven coined the term 'metabolic syndrome' and subsequently renamed it the 'insulin resistance syndrome' [1]. However, it becomes increasingly clear that we could better refer to it as the 'chronic systemic low-grade inflammation induced energy reallocation syndrome'. The reason for this broader name derives from the recognition that insulin resistance is only part of the many simultaneously occurring adaptations. To their currently known extent, these adaptations and consequences are composed of: i) reduced insulin sensitivity (glucose and lipid redistribution, hypertension), ii) increased sympathetic nervous system activity (stimulation of lipolysis, gluconeogenesis and glycogenolysis), iii) increased activity of the HPA-axis [hypothalamus-pituitary-adrenal gland (stress) axis, mild cortisol increase, gluconeogenesis, with cortisol resistance in the immune system], iv) decreased activity of the HPG-axis (hypothalamus-pituitary-gonadal gland axis; decreased androgens for gluconeogenesis from muscle proteins, sarcopenia, androgen/estrogen disbalance, inhibition of sexual activity and reproduction), v) IGF-1 resistance (insulin-like growth factor-1; no investment in growth) and vi) the occurrence of 'sickness behavior' (energy-saving, sleep, anorexia, minimal activity of muscles, brain, and gut) [3].

The HPT-axis (hypothalamic-pituitary-thyroid axis) has a central role in our energy management. The adaptation of thyroid function in subjects with the metabolic syndrome is yet unclear, possibly due to the

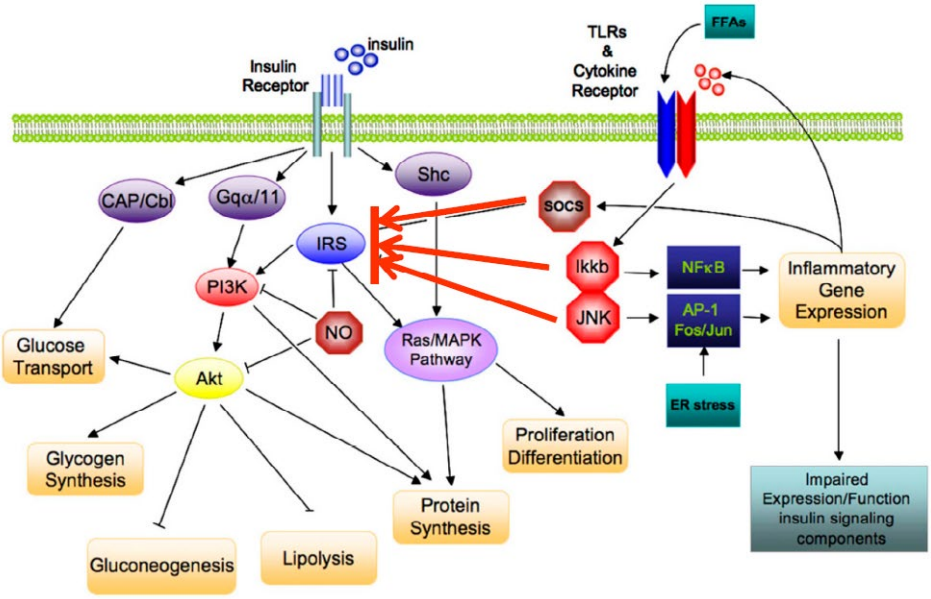

Figure 4. Mechanistic connection between inflammation and insulin resistance.

The NFκB and AP-1 Fos/June inflammatory pathways inhibit the PI3K/AKT signal transduction pathway for nutrient metabolism and the Ras/MAPK pathway for gene expression, both part of the insulin signaling. CAP, Cbl associated protein; Cbl, Proto-oncogene product; ER, endoplasmic reticulum; FFAs, Free fatty acids; Gqα/11, heterotrimeric g protein; Ikkb, I kappa B kinase Beta; IRS, insulin receptor substrate; JNK, C-jun N-terminal kinase; NFkB, nuclear factor kappa B; NO, nitric oxide; Ras/MAPK; PI3K, phosphatidylinositol 3-kinase; Ras-mitogen activated protein kinase; Shc, Src homology 2 containing protein; SOCS, supressor of citokyne signaling; TLRs, Toll-like receptors. Adapted from de Luca and Olefsky [48] with permission from Elsevier.

many concerted changes, such as an altered thyroid hormone binding capacity, tissue uptake, conversion of T_4 into T_3, and tissue-specific receptor expression and function. For example, T_4 may become converted into the highly active T_3 within the target cell and thereby, without visible changes of circulating hormone concentrations, bind to the intracellular thyroid hormone receptor [55]. Whether intracellular T_4 is converted into T_3 or the inactive reverse T_3 (rT_3), or is used as a source of iodine to kill bacteria, depends on several factors, including cytokines, that determine the expression pattern of the three involved deiodinases [55–57]. In euthyroid subjects, free T_4 (FT_4) is associated with insulin resistance, inversely related to total- and LDL-cholesterol, while also a positive relationship between TSH and triglycerides has been documented [58]. The reported changes during metabolic syndrome [59], low-grade inflammation and insulin resistance [60] are inconsistent, but do bear great resemblance with subclinical hypothyroidism, with high-normal or slightly elevated TSH, and normal FT_4 concentrations [61, 62]. Insulin resistance has recently been associated with an increased T_3/rT_3 ratio, which is a measure of peripheral thyroid hormone metabolism and suggests increased thyroid hormone activity [63]. In contrast, during fasting, energy expenditure becomes downregulated, resulting in a normal or decreased TSH and decreased serum thyroid hormone concentrations [64]. Downregulation of the HPT-axis with reductions of T_3, T_4 and TSH, and an increase of rT_3 (and thus a decrease of the T_3/rT_3 ratio) occurs progressively with the severity of the 'non-thyroidal illness syndrome' (NTIS, also called the 'Low T_3 syndrome' and 'euthyroid sick syndrome') [55] which is explained as an adaptation of the body to prevent excessive (protein) catabolism as part of the acute phase response [56].

All of the above mentioned adaptations of our metabolism are associated with changes in the serum lipoprotein profile, which are part of the metabolic syndrome. The purpose of these changes will be explored in more detail below.

Changes in serum lipoproteins

The quantitative and qualitative changes in the composition of serum lipoproteins resulting from an inflammatory trigger have, in addition to the reallocation of energy-rich nutrients (fatty acids to the insulin resistant organs), at least two other goals [5–10, 65]. These are: i) the modulation of the immune response by which we protect ourselves from the harmful effects of invading bacteria, viruses and parasites, and ii) the restoration of the hereby inflicted damage. However, if the subsequent changes in structure and function of lipoproteins persist, they contribute to the development of atherosclerosis [66]. These long term complications have not exerted selection pressure during evolution and, consequently, no solution has come into existence via the habitual process of spontaneous mutation and natural selection.

The inflammatory trigger during an infection with Gram-negative bacteria is initiated by lipopolysaccharides (LPS). Circulating lipoproteins aid in the clearance of this LPS. Hence, lipoproteins do not only have functions in transporting lipids to and from tissues, but also play important roles in limiting the inflammatory response [67]. The ability of lipoproteins to bind LPS is proportional to the cholesterol content of the lipoprotein [68], but the phospholipids/cholesterol ratio of the lipoprotein is the principal determinant of the LPS-binding capacity [69]. The available phospholipid surface is thus of special importance and is, under normal circumstances, the largest for the circulating HDL. However, critically ill patients exhibit decreases of both esterified cholesterol and HDL (see below) and in those patients, LPS is mainly taken up in the phospholipid layers of LDL and VLDL. Binding of LPS to lipoproteins prevents activation of LPS-responsive cells and encourages LPS clearance via the liver to the bile. In line with this mechanism, it has been observed that a decrease in plasma lipoproteins in experimental models increases LPS-induced lethality [69].

The protective role of LDL is already known for some time, and this process has probably been exploited during evolution. Currently, there are over one thousand LDL-receptor mutations, many of which lead to a reduced or absent hepatic uptake of LDL particles, and consequently, to an elevated serum LDL-cholesterol [70]. The carriers of these mutations have 'familial hypercholesterolemia' (FH; incidence about 1/400 in The Netherlands) or 'defective apo-B100' (FDB), if the mutation is located in the LDL-receptor ligand. They constitute autosomal dominant disorders with a high risk of premature atherosclerosis and mortality from CVD [71]. The arising question is why evolution has preserved so many apparently detrimental mutations in the LDL-receptor. Research with data from the population registry office in The Netherlands showed that subjects with FH lived longer until 1800, which turned into a shorter lifespan than the general population after 1800 [72]. Important support for an explanation came from studies with LDL-receptor knockout mice, and also with transgenic mice overexpressing apo-A1, the structural protein of HDL. These mutants have a high LDL- and HDL-cholesterol, respectively, are resistant to LPS-induced mortality, and have better survival of severe Gram-negative infection compared with the wild type [66, 73]. In other words, FH might have become widespread during evolution due to the modulating effect of a high LDL (i.e. 'a high cholesterol') during Gram-negative infections, that were much more common in the past. This benefit might have become a risk following the introduction of a typically Western lifestyle (see below), to which subjects with FH seem particularly sensitive [72].

As mentioned above, among the lipoproteins, notably HDL has the capacity to bind LPS and thereby to prevent an LPS-induced activation of monocytes and the subsequent secretion of proinflammatory cytokines [5]. However, during the 'lipidemia of sepsis', the HDL concentration decreases while also the HDL particles decrease in size [6]. Their function changes as part of the acute phase response: the immunomodulatory properties vanish to a high extent and HDL even becomes proinflammatory. The apo-A1 and cholesterol esters are lost from the HDL particle, the activities of HDL-associated enzymes and exchange proteins decrease, and these proteins are, among others, replaced by serum amyloid A (SAA) [5, 6]. Like CRP, SAA is produced in the liver as part of the acute phase response. SAA is 90% located in HDL, prevents the uptake of cholesterol by the liver and directs it to other cells such as macrophages [8, 66]. Both the decreasing HDL-cholesterol and the concomitantly reduced 'cholesterol reverse transport', promote the accumulation of cholesterol in the tissues, where it is needed for the synthesis of steroid hormones (e.g. cortisol) in the adrenal glands, the immune system and for the synthesis of cellular membranes that became damaged by the infection [66]. Also the formation of small dense LDL [74] might be functional because these particles are poorly cleared by the

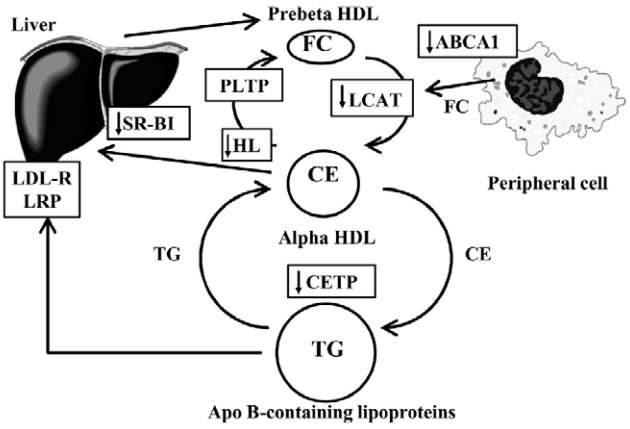

Figure 5. Changes in reverse cholesterol transport during the acute phase response.

Lipopolysaccharides (LPS) and cytokines reduce the ABCA1 (ATP binding cassette transporter A1) and the cholesterol efflux from peripheral cells to HDL. LPS reduces the activities of various proteins involved in HDL metabolism, such as lecithin-cholesterol acyltransferarse (LCAT), cholesterol ester transfer protein (CETP) and hepatic lipase (HL). LPS and cytokines also down-regulate hepatic scavenger receptor class B type 1 (SRB1), resulting in a decreased cholesterol ester (CE) uptake in the liver. FC, free cholesterol; LDL-R, LDL receptor; LRP, LDL receptor-related protein; PLTP, phospholipid transfer protein. Adapted from Khovidhunkit et al. [66] with permission from The American Society for Biochemistry and Molecular Biology.

LDL-receptor, easily penetrate the subendothelial space and by their binding to the subendothelial matrix, take their cholesterol cargo to the sites of damage in a highly efficient manner. It appears that there are numerous mechanisms that jointly cause the active inhibition of the reverse cholesterol transport in response to an acute phase response (Figure 5) [66, 75].

Summarizing thus far, we humans are extremely sensitive to glucose deficits, because our large brain functions mainly on glucose. During starvation, pregnancy and infection/inflammation, we become insulin resistant, along with many other adaptations. The goal is the reallocation of energy-rich substrates to spare glucose for the brain, the rapidly growing infant during the third trimester of pregnancy, and our activated immune system that also functions mainly on glucose. Under these conditions, the insulin resistant tissues are supplied with fatty acids. Other goals of the changes in the serum lipoprotein composition are their role in the modulation of the immune response by the clearance of LPS during infection/inflammation and the redirection of cholesterol to tissues for local damage repair. The metabolic adaptations caused by inflammation illustrate the intimate relationship between our immune system and metabolism. This relation is designed for the short term. In a chronic state it eventually causes the metabolic syndrome and its sequelae. We are ourselves the cause of the chronicity. Our current Western lifestyle contains many false inflammatory triggers and is also characterized by a lack of inflammation suppressing factors. These will be described in more detail below.

Lifestyle-induced chronic systemic low grade inflammation

An inflammatory reaction is the reflection of an activated immune system that aims to protect us from invading pathogens or reacts to a sterile infection. If an activated immune system is uncontrolled, the resulting secondary reactions have the ability to kill us. Rogers [76] expresses it as follows: '...inflammation may be useful when controlled, but deadly when it is not. For example, head trauma may kill hundreds of thousands of neurons, but the secondary inflammatory response to head trauma may kill millions of neurons or the patient'. It is clear that an inflammatory reaction that has started should subsequently be ended.

There are many factors in our current Western lifestyle that jointly cause a state of chronic systemic low grade inflammation, which in turn leads to chronically compromised insulin sensitivity, compensatory hyperinsulinemia and, eventually, the diseases related to the metabolic syndrome. Lifestyle factors that cause inflammation can be subdivided into an unbalanced composition of the diet (usually referred to as 'malnutrition') [78–80] and non-food related factors [77], which partly exert their influence via obesity [81] (Table 1). Among the pro-inflammatory factors in our current diet, we find: the consumption of saturated fatty acids [82] and industrially produced trans

Table 1. Environmental factors that may cause chronic systemic low grade inflammation.

Adapted from Egger and Dixon (77).

Pro-Inflammatory				Anti-Inflammatory			
Lifestyle	Exercise	too little (inactivity)		Lifestyle	Exercise/physical activity/fitness		
		too much					
	Nutrition	alcohol (excessive)			Nutrition	alcohol	
		excessive energy intake				energy intake (restricted)	
		starvation					
		'fast food'/ Western style diet				Mediterranean diet	
		fat	high-fat diet			fat	fish/fish oil
			saturated fats				mono-unsaturated fats
			trans fatty acids				olive oil
			high ω6/ω3 ratio				low ω6/ω3 ratio
		fiber (low intake)				fiber (high intake)	
		fructose				nuts	
		glucose	high glucose/GI foods			low GI foods	
			glycemic load			grapes/raisins	
			glycemic status			dairy calcium	
			sugar-sweetened drinks			eggs	
		meat (domesticated)				lean meats (wild)	
		salt				soy protein	
						fruits/vegetables	
						cocoa/chocolate (dark)	
						herbs and spices	
						tea/green tea	
						capsaicin (pepper)	
						garlic	
						pepper	
	Obesity				'Healthy obesity'		
	Weight gain				Weight loss		
	Smoking				Smoking cessation		
	'Unhealthy lifestyle'				Intensive lifestyle change		
	Stress/anxiety/depression/burn out						
	Sleep deprivation						
Age							
Environment	Socioeconomic status (low)						
	Perceived organizational injustice						
	Air pollution (indoor/outdoor)						
	Second-hand smoking						
	'Sick building syndrome'						
	Atmospheric CO_2						

fatty acids [83, 84], a high ω6/ω3 fatty acid ratio [85–87], a low intake of long-chain polyunsaturated fatty acids (LCP) of the ω3 series (LCPω3) from fish [88, 89], a low status of vitamin D [90–92], vitamin K [93] and magnesium [94–96], the 'endotoxemia' of a high-fat low-fiber diet [97, 98], the consumption of carbohydrates with a high glycemic index and a diet with a high glycemic load [99, 100], a disbalance between the many micronutrients that make up our antioxidant/pro-oxidant network [101–103], and a low intake of fruit and vegetables [103, 104]. The 'dietary inflammation index' of the University of North Carolina is composed of 42 anti- and proinflammatory food products and nutrients. In this index, a magnesium deficit scores high in the list of pro-inflammatory stimuli [105]. Magnesium has many functions, some of them, not surprisingly, related to our energy metabolism and immune system, e.g., it is the cation most intimately connected to ATP [95]. Indirect diet-related factors are an abnormal composition of the bacterial flora in the mouth [106], gut [106, 107], and gingivae [108–110]. Chronic stress [111, 112], (passive) smoking and environmental pollution [77], insufficient physical activity [113–118] and insufficient sleep [119–123] are also involved.

All of the above listed lifestyle factors exhibit interaction and are therefore difficult to study in isolation. As an example, the bacterial flora may change secondary to the composition of our diet. An inflammatory reaction might be at the basis of the observed relation between the abnormal bacterial species in both our oral cavity and intestine and our serum HDL- and LDL-cholesterol [106]. Saturated fats may cause an inflammatory reaction especially when they are combined with a carbohydrate-rich diet, notably carbohydrates with a high glycemic index, and especially in subjects with the insulin resistance syndrome [124–128].

1

Mechanisms of lifestyle-induced inflammation

Diets high in refined starches, sugar, saturated and trans fats, and low in LCPω3, natural antioxidants, and fiber from fruits and vegetables, have been shown to promote inflammation [82–84, 129–131] (Table 1). As most chronic (inflammatory) diseases have been linked to diet, modifying it could prevent, delay or even heal these diseases. Obviously. inflammation is an essential process for survival, but our immune system should be carefully controlled to limit the unavoidably associated collateral damage [132]. For instance, wound healing and other immune challenges become controlled in our body by a process coined by Serhan et al. [133–135] as *resoleomics*, using metabolites produced from the LCP arachidonic acid (AA), EPA and DHA [85, 133–136]. However, our inflammatory and resolution genes operate nowadays in a completely different environment than the one to which they became adapted through mutation and natural selection. In most (if not all) chronic diseases typical of Western societies, the inflammatory response is not concluded because of suboptimal or supramaximal responses [137, 138].

It has been estimated that 10% of all deaths in the Netherlands are attributable to unfavorable dietary composition and 5% to overweight. In this scenario, the major contributors to diet-associated death were insufficient intakes of fish, vegetables and fruits, with less important roles for too high intakes of saturated and *trans* fatty acids [139]. The consumption of fish, fruit and vegetables is considered too low in most Western countries [139–143]. In the USA, low dietary ω3 fatty acids and high dietary *trans* fatty acids may have accounted for up to 84,000 and 82,000 deaths, respectively, in 2005, while a low intake of fruit and vegetables might have been responsible for 58,000 deaths [144]. The Dutch [145] and the American Heart Association (AHA) [146] dietary guidelines recommend to consume at least two servings of fish per week (particularly fatty fish), but in 1998, the average fish consumption in The Netherlands amounted to hardly 3 times per month [139]. Only about 7% of the 9–13 year-old Dutch children eat fish twice or more per week and 10% never eat fish [147]. In the USA, the estimated intake of fish in 2007 was about 0.7 kg per month, per person. More preoccupying is the fact that the USA is considered the third largest consumer of seafood in the world [148, 149]. Despite improvements of the fatty acid contents of food products, only 5% of the Dutch population follows a diet with the recommended fatty acid pattern [139]. Eating fish once weekly was associated with a 15% lower risk of CVD death compared with a consumption of less than once per month [150], while each 20 g/day increase in fish consumption was related to a 7% lower risk of CVD mortality [151].

The current Dutch recommendation for adults is 200 g fruits and 200 g vegetables per day [139], while in the USA, 4–5 servings of fruits and 4–5 servings of vegetables are recommended in a 2,000 kcal diet [152]. Between 1988 and 1998, the consumption of fruit and vegetables in The Netherlands declined 15–20% and currently, less than 25% of the Dutch population follows the recommendations regarding the consumption of fruit, vegetables and dietary fiber [139]. As an example, currently 99% and 95% of the 9–13 year old Dutch do not adhere to the advice of consuming 150 g/day vegetables and 200 g/day fruits, respectively [147]. Meta analyses of prospective studies indicated that <3 vs. >5 servings of fruits and vegetable per day correspond with a 17% reduction in coronary heart disease [153] and 26% reduction in stroke [154], while the relation of low intakes with mouth, pharynx, esophagus, lung, stomach, colon and rectum cancer is considered substantially convincing [155].

In view of the numerous nutrients present in our food and their many mechanisms of action in the inflammatory response, we selected two nutrient classes, i.e. the LCP from fish (LCPω3; notably EPA and DHA), and the antioxidants in fruit and vegetables, to illustrate the many dietary components involved in our pro-inflammatory/anti-inflammatory balance. However, before embarking into these nutrient classes, it should be emphasized that our food is in reality composed of biological systems, such as meat, fish, vegetables and fruits, in which nutrients obey to the balance that comes along with living material. Therefore, focusing on specific, presently known mechanisms without sufficient knowledge of the many possible interactions between the numerous nutrients in our food should be regarded as a serious limitation. This is a reductionist approach, whereas system dynamics and holistic approximations would be more appropriate.

Fatty acids and inflammation

The media are consistently reporting on advises to reduce fat consumption to avoid risks associated with obesity, CVD, diabetes and other chronic diseases and conditions. Among the macronutrients, fat does

indeed contain the highest amount of energy per gram. However, from a thermodynamic point of view, a 'calorie is a calorie' [156], implying that any macronutrient consumed in disbalance with energy expenditure and thermogenesis might cause obesity. A recent in-depth study revealed that 'a calorie is not a calorie' in a metabolic sense, showing that isocaloric diets with different macronutrient compositions have different effects on resting and total energy expenditure with decreasing energy expenditures in the sequence low-fat diet<low-glycemic diet<very low-carbohydrate diet [157], and thereby suggesting that the diet with the highest protein and fat content gives rise to the lowest weight gain. However, whether the intake of fat *per se* and, as a matter of fact, any isolated nutrient [158], can be held responsible for the epidemics of obesity, remains controversial and counter intuitive [159–161]. Moreover, it is becoming increasingly clear that about 10–25% of obese subjects have little CVD and type 2 diabetes mellitus risk (a condition coined 'healthy obesity') [162, 163], that lean physically unfit subjects have higher risk of CVD mortality than obese, but fit, subjects [164], and that it is the quality and not the quantity of fat that conveys a major health hazard [165]. The type of dietary fat affects vital functions of the cell and its ability to resist disfunction e.g. by influencing the interaction with receptors, by determining basic membrane characteristics and by producing highly active lipid mediators [166, 167].

Saturated fat intake has been associated with inflammation [168, 169]. However, the widely promoted reduction of saturated fatty acids is increasingly criticized [170] and also the AHA advisory to replace saturated fatty acids in favor of linoleic acid (LA) to 5–10 en% [171]. Insufficient intake of particular fatty acids is, on the other hand, likely to contribute to health hazards, including increased risk of infection [172], dysregulated chronobiological activity and impaired cognitive and sensory functions (especially in infants) [173]. Among these important fatty acids are the LCPω3 derived from fish, of which EPA and DHA are the most important members. In 2003, the intake of EPA+DHA by adults in The Netherlands amounted to approximately 90 mg/day (women 84 mg/day and men 103 mg/day) [174], while the recommendation is 450 mg/day [175]. This recommendation is based on an optimal effect in preventing CVD (anti-arrhythmic effect), but there is good evidence that higher intakes may convey additional favorable effects because of their anti-thrombotic properties and their ability to reduce blood pressure, heart rate and triglyceride levels [131]. It was calculated that our Paleolithic ancestors living in the water-land ecosystem had daily intakes of 6–14 g EPA+DHA [176], which correspond with the intakes by traditionally living Greenland Eskimos [177], who, because of their low incidence of CVD, were at the basis of the research on the beneficial effects of fish oil that started in the seventies [178–180].

Both EPA and DHA must be in balance with AA, which is the major LCPω6 derived from meat, poultry, eggs [181–183] and also lean fish [184, 185]. Each of these LCP may be synthesized by desaturation, chain elongation and chain shortening from the parent 'essential fatty acids' LA (converted to AA) and alpha-linolenic acid (ALA) (converted to EPA and DHA) [186], even though the production of EPA, and notably DHA, occurs with difficulty in humans [187]. Included among the symptoms of LA, LCPω3 and LCPω6 deficiencies are fatigue, dermatological problems, immune problems, weakness, gastrointestinal disorders, heart and circulatory problems, growth retardation, development or aggravation of breast and prostate cancer, rheumatoid arthritis, asthma, preeclampsia, depression, schizophrenia and ADHD [173, 188–190].

LCPω3 are implicated in many diseases and conditions, including CVD, psychiatric diseases, pregnancy complications and suboptimal (neuro) development [86, 191–196]. Moreover, a growing number of studies indicate the protective effects of dietary LCPω3 on mood symptoms, cognitive decline, depression [197, 198], Alzheimer's disease [199] and, more generally, impaired quality of life both in the elderly [200, 201] and younger [202] populations. LCPω3 are involved in numerous processes including energy generation, growth, cell division, transfer of oxygen from the air to the bloodstream, hemoglobin synthesis, normal nerve impulse transmission and brain function. Many different mechanisms are operational: LCPω3 mediate potent anti-inflammatory and insulin sensitizing effects through their interaction with a membrane receptor named G-protein-coupled receptor 120 (GPR120) [203, 204]; they act at the gene expressional level by binding to nuclear receptors, such as the peroxisome proliferator activated receptors (PPARs) [205–207]; and they modulate physical and metabolic properties of membranes through their incorporation into phospholipids and thereby impact on the formation of lipid raft [134, 208, 209]. Important common denominators in each of these interactions seem to be their anti-inflammatory and metabolic effects,

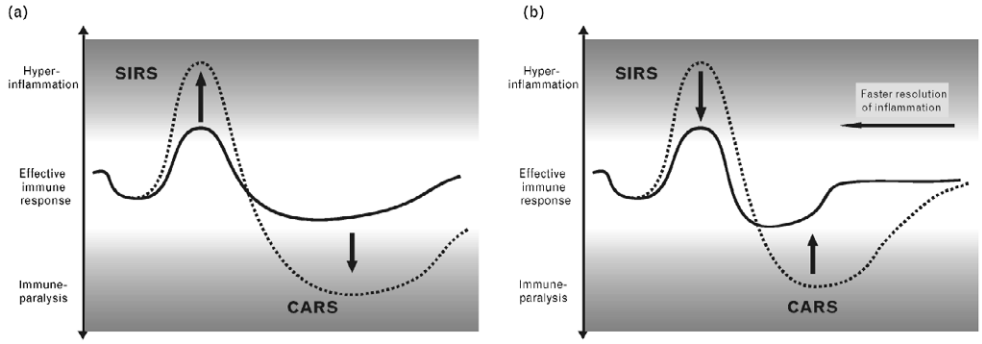

Figure 6. LCPω6 and LCPω3 postulated involvement in the inflammatory reaction in sepsis and its subsequent resolution.
Sepsis causes a systemic inflammatory response giving rise to the 'systemic inflammatory response syndrome' (SIRS). The inflammatory response is followed by a compensatory anti-inflammatory response, which results in the 'e' (CARS), characterized by a weakened host defense and augmented susceptibility to secondary infections. An inflammatory response should not only be initiated, but also stopped to limit collateral damage produced by the immune system and to prevent immune paralysis. LCPω6 (AA) are involved in the initiation of the inflammatory reaction, while LCPω3 (EPA and DHA) are involved in its resolution (see also Figure 7). a) A high LCPω6/LCPω3 ratio, e.g. low fish intake, intensifies the SIRS reaching a state of hyper-inflammation, while the CARS leads to a state of immune paralysis. b) A low LCPω6/LCPω3 ratio dampens both the SIRS and CARS, resulting in a more balanced immune response and preventing hyper-inflammation and immune-paralysis. SIRS, systemic inflammatory response syndrome; CARS, compensatory anti-inflammatory response syndrome. Adapted from Mayer et al. [220] with permission from Wolters Kluwer Health.

again illustrating the intimate connection between the immune system and metabolism [50, 51].

The modernization of food manufacturing, preservation processes and food choices have dramatically altered the balance between LCPω3 and LCPω6 in the Western diet, notably by increasing the intake of LA from refined vegetable oils and a concomitant decrease in the intake of LCPω3 from fish [210, 211]. It is gaining acceptance that it is not the amount of fat but the balance between the different types of fatty acids that is important [211, 212]. A high ω6/ω3 fatty acid ratio has been demonstrated to have an inflammatory effect [86, 212, 213], while a higher intake of LCPω3 in the form of EPA and DHA regulates the production of inflammatory and resolving cytokines and decreases LA levels in both plasma phospholipids and cell membranes [183, 214]. The conversions of LA and ALA to AA and to EPA+DHA, respectively, depend on the same enzymes in the desaturase and elongase cascade, with Δ6-desaturase (FADS2) as a rate-limiting enzyme [215] that functions twice in the biosynthesis of DHA [216]. Increased consumption of ALA gives rise to an increased ALA/LA ratio and EPA+DHA content in cell membranes that comes together with a reduction of the AA content [216, 217], and thereby influences the balance between inflammation and its subsequent resolution (Figure 6) [218–220]. Conversely, a higher LA level in plasma phospholipids and cell membranes emerges as a major factor responsible for incomplete *resoleomics* reactions and the associated immune paralysis [214, 220, 221] (Figure 6), which is attributed to the competitive inhibition of LA in the conversion of ALA to EPA and DHA and also to the competition of LA in the incorporation of EPA and DHA into cellular phospholipids [183, 214, 216].

LCPω3 and LCPω6 have distinct functions in the inflammatory reaction and its resolution. In the first phase of the inflammatory process, the pro-inflammatory eicosanoids leukotrienes-B_4 (LTB_4) and prostagladins-E_2 and D_2 (PGE_2 and PGD_2) [222, 223] are generated by macrophages from their precursor AA with the help of the lipid-oxidizing enzyme lipoxygenase-5 (LOX-5) and cyclo-oxygenase-2 (COX-2) [224–226]. At the same time, PGE_2 and/or PGD_2, although initially pro-inflammatory, determine the switch to the next phase: the resolution of the inflammation [227] via the so-called 'eicosanoid-switch'. The production of the LOX-5 enzyme becomes limited, while anti-inflammatory lipoxins (LXs) are produced from AA through the activation of lipoxygenase-12 (LOX-12), lipoxygenase-15 (LOX-15) and acetylated COX-2 [228]. At the site of inflammation, LOX-12 produced by platelets

converts LTA_4 to LXA_4 and LXB_4. Along with AA, both LOX-12 and -15 are involved in the biosynthesis of specialized bioactive lipid mediators, coined resolvins, (neuro)protectins [135] and maresins [229], which derive from EPA and DHA (Figure 7) [85, 134, 172]. Several studies have illustrated the involvement of these lipid mediators in vascular inflammation and atherosclerosis [85, 228, 230, 231]. They possess potent anti-inflammatory and pro-resolving actions that stimulate the resolution of acute inflammation by reducing and/or limiting the production of a large proportion of the pro-inflammatory cytokines produced by macrophages. Furthermore, LXA_4, protectin D1 and resolvin D1 stimulate the phagocytic activity of macrophages toward apoptotic cells and inhibit inflammatory cell recruitment [232, 233] thereby protecting tissues from excessive damage by the oxidative stress that comes along with immune defense mechanisms and others. By their inhibitory actions on the recruitment of inflammatory cells, they allow the resolution phase to set in [234] and finish the inflammatory process with the return to homeostasis [136, 227].

Accordingly, LCPω3 given at doses of hundreds of milligrams to grams per day, exhibits beneficial actions in many inflammatory diseases [88, 190, 194, 235, 236]. For example, DHA has been shown to suppress NFκB activation and COX-2 expression in a macrophage cell line [168, 237]. Different studies demonstrated the nutrigenetic modulation of the 12/15-LOX by providing endogenous anti-inflammatory signals and protection during the progression of atherogenesis [231, 238, 239], which seem to be totally annulled in the presence of Western diet induced hyperlipidemia. As some eicosanoids regulate the production of inflammatory cytokines [85, 134, 135] an LCPω3-induced decrease in pro-inflammatory eicosanoid production might affect the production of pro-inflammatory cytokines. Equally important is the observation that LCPω3 also modulate the activation of transcription factors involved in the expression of inflammatory genes (e.g. NFκB, phosphatidylinositol 3-kinase (PI3K)) [240]. Hence, a high fish consumption, and especially fatty fish, rich in EPA and DHA, seems of crucial importance in the primary and secondary prevention of (Western) chronic diseases [241, 242], although it should be emphasized that fish is not a synonym of fish oil and also that insufficient fish consumption is certainly not the only factor involved in the pro-inflammatory Western lifestyle (Table 1).

Role of the antioxidant network

The largest contributor to mortality and morbidity worldwide is age-related, non communicable disease, including cancer, CVD, neurodegenerative diseases and diabetes [244]. Even though these are multi-factorial diseases with many pathophysiological mechanisms, a common finding is oxidation-induced damage through oxidative stress [245, 246]. Appropriate antioxidant intake has been proposed as a solution to counteract the deleterious effects of reactive oxygen species (ROS; e.g. hydrogen peroxide, hypochlorite anion, superoxide anion and hydroxyl radical), with substantial evidence upholding the contention that: a diet rich in natural antioxidants supports health [104, 246], is associated with lower oxidative stress and inflammation [77, 103, 140], and is therefore associated with lower risk of cancer, CVD, Alzheimer's disease, cataracts, and some of the functional declines associated with aging [247–251].

Molecular oxygen is essential to aerobic life and, at the same time, an oxidizing agent, meaning that it can gain electrons from various sources that thereby become 'oxidized', while oxygen itself becomes 'reduced' [252, 253]. In general terms, an antioxidant is 'anything that can prevent or inhibit oxidation' and these are therefore needed in all biological systems exposed to oxygen [252]. The emergence of oxygenic photosynthesis and subsequent changes in atmospheric environment [254] forced organisms to develop protective mechanisms against oxygen's toxic effects [255]. Change is implicit to evolution and evolution results in adaptation to change [256]. As a result, many enzymatic reactions central to anoxic metabolism were effectively replaced in aerobic organisms and antioxidant defense mechanisms evolved [257, 258]. The continuous exposure to free radicals from a variety of sources led organisms to develop a series of systems [259] acting as a balanced and coordinated network where each one relies on the action of the others [260, 261].

Oxidative stress occurs when there is a change in this balance in favor of ROS [262] that may occur under several circumstances, ranging from malnutrition to disease [263, 264]. Damage by oxidation of lipids [262, 265, 266], nucleic acids and proteins changes the structure and function of key cellular constituents resulting in the activation of the NFκB pathway, promoting inflammation, mutation, cell damage and even death [252, 260, 267], and is thereby believed to underlie the deleterious changes in aging and

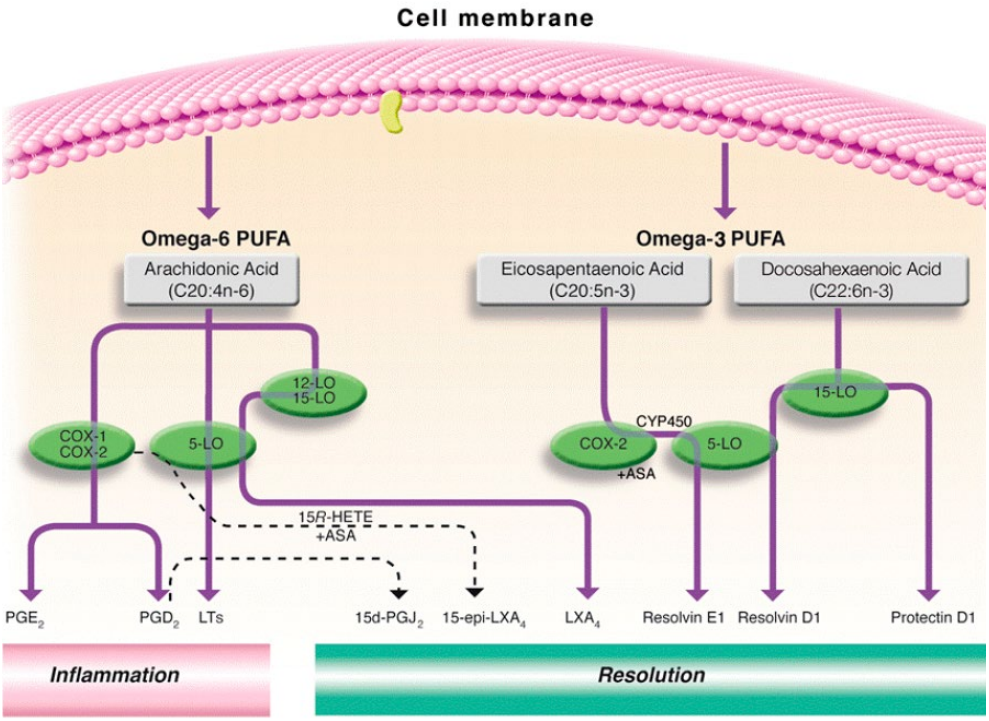

Figure 7. Biosynthesis of inflammatory and resolving lipid mediators.

AA is released from membrane phospholipids by phospholipase A_2 (PLA_2) and metabolized by COXs or 5-LO to form inflammatory mediators, such as prostaglandins and leukotrienes. During the process of resolution, there is a 'switch' from the biosynthesis of inflammatory mediators to the formation of lipid derivatives with anti-inflammatory and pro-resolving properties, including lipoxins and 15-d-PGJ_2. EPA and DHA are converted to potent anti-inflammatory and pro-resolving lipid mediators like resolvins (E1 and D1) and protectins. ASA, acetylsalicylic acid, CYP450, cytochrome P450, COX-1, cyclo-oxygenasa-1, COX-2, cyclo-oxygenasa-2; 5-LO, 5-lipo-oxygenase; 12-LO, 12-lipo-oxygenase; 15-LO, 15-lipo-oxygenase; PGE_2, prostagladin-E_2; PGD_2, prostaglandin-D_2; LTs, leukotrienes; 15d-PGJ_2,15-deoxy-delta-12,14-prostaglandin J_2; 15-epi-LXA_4, 15-epi-lipoxin A_4; LXA_4, lipoxin A_4. Adapted from González-Périz and Clària [243] with permission.

Table 2. Types of antioxidant action.

	Action	Examples
Prevention	Protein binding/inactivation of metal ions	Transferrin, ferritin, ceruloplasmin, albumin
Enzymatic Neutralization	Specific channelling of ROS into harmless products	SOD, catalase, glutathione peroxidase
Scavenging	Sacrificial interaction with ROS by expendable (recyclable or replaceble) substrates	Ascorbic acid, alpha tocopherol, uric acid, glutathione
Quenching	Absorption of electrons and/or energy	α-tocopherol, β-carotene, astaxanthin

ROS, reactive oxygen species; SOD, superoxide dismutase. Adapted from Benzie [260].

age-related diseases [102, 244]. The prevention and/or inhibition of oxidation can be achieved by several types of specialized antioxidant mechanisms depicted in Table 2 [260]. Our antioxidant system is composed of two networks (Figure 8), namely, the antioxidant network of non-enzymatic antioxidants that we obtain mostly via the diet [268], and the antioxidant enzymes that we synthesize ourselves and that carry metal ions for their appropriate functioning in ROS clearance. Members of the non-enzymatic antioxidants are e.g. ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), carotenoids, and the polyphenols [269, 270]. For instance, quercetin, one of the most common flavonoids in the human diet, and resveratrol, a well-known stilbenoid present mostly in berries and the skin of red grapes, have demonstrated favorable effects on glucose metabolism by attenuating TNFα-mediated inflammation and insulin resistance in primary human adipocytes [271]. Typical examples of the antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [252].

While the prevention of oxidative stress by enhancing the antioxidant defense mechanisms may diminish the production of inflammatory mediators and thereby slow aging and lower risk of certain diseases [102, 245, 249], it should at the same time be appreciated that ROS also exert essential metabolic and immune functions. For example, oxidative phosphorylation is based on electron transport [272], which renders free radicals' inevitable byproducts of mitochondrial metabolism [273]. Mitochondrial oxidants may function as signaling molecules in the communication between the mitochondria and the cytosol [273], while TNFα-induced apoptosis may involve mitochondria-derived ROS [274]. The innate immune system kills microbes by means of the respiratory burst [275]. A certain level of ROS may also be essential to trigger antioxidant responses [276]. Repeated exposure to sublethal stress has been proposed to result in enhanced stress resistance and increased survival rates, which in the dose-response curve is better known as hormesis [277]. Intracellular ROS may stimulate gene expression of antioxidant and immunoreactive proteins [278], while SOD may become upregulated in chronic exercise through the binding of NFκB to the SOD promoter [279, 280].

Consequently, certain antioxidants may inhibit mitochondrial biogenesis, interfere with the hormetic effects of ROS [281, 282] or have other adverse effects. Effective prevention of ROS formation and their removal may therefore upset energy metabolism, cell signaling pathways and the immune system, and thereby paradoxically increase the risk of chronic disease [283]. Moreover, any antioxidant is also a potential pro-oxidant because in its scavenging action it gains an extra electron that can initiate a new radical reaction when transferred to an acceptor, either spontaneously or upon decomposition [284, 285]. Possibly through its prooxidant action or other mechanisms [286], meta-analyses of studies with β-carotene dosages above 20 mg/day have shown increased risk of lung cancer in the total population, smokers and asbestos workers; and of stomach cancer in smokers and asbestos workers [287]. Analogously, oral antioxidants to limit muscle damage following exercise training may be detrimental to health and performance [288], while β-carotene, vitamin A and vitamin E supplements have been connected with higher risk of all-cause mortality [289], although the outcome of the latter meta-analysis has been contested [290]. Moreover, not all antioxidants are created equal. Astaxanthin, a carotenoid from the land-water ecosystem, does not appear to exhibit pro-oxidant properties [291] when supplemented alone, even at high doses [292], and has been shown to decrease oxidative stress and inflammation in various circumstances [266, 293].

Chronic inflammation results in the chronic generation of free radicals, which may cause collateral damage and stimulate signaling and transcription factors associated with chronic diseases [294, 295]. The hypothesis that dietary antioxidants lower the risk of chronic diseases has been developed from epidemiological studies consistently showing that consumption of fruit and vegetables is strongly associated with a reduced risk of these diseases [104, 248, 250]. Regular consumption of green tea [296] and red wine [103, 297], both rich in polyphenols, decreases DNA damage, and the same holds for the kiwifruit [298] and watercress [299], both harboring high amounts of carotenoids and vitamin C. On a calorie basis, fruits and vegetables are not only richer in many vitamins and minerals, when compared with cereals, meat or fish, but also in antioxidants [300]. These may collectively be responsible of the aforementioned protection of fruits and vegetables in chronic diseases, including CVD [248] and cancer [249]. Plants harbor similar defense mechanisms as animals for protection against ROS [301]. Some of their antioxidants are part of their arsenal of 'secondary metabolites', defined as those

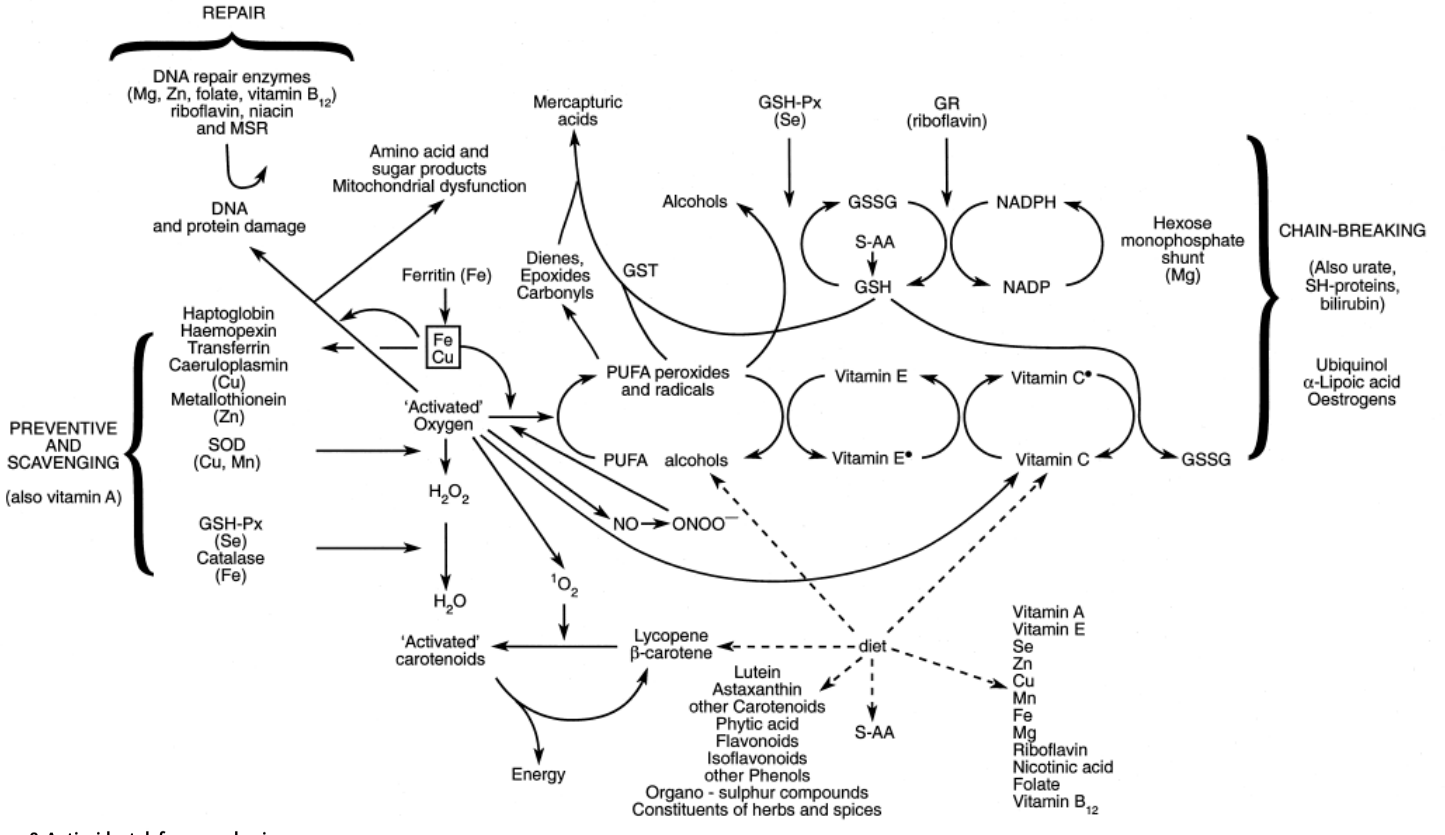

Figure 8. Antioxidant defense mechanisms.

An overview of the antioxidant system present in the human body. Various types of antioxidant systems have developed through time, reflecting different selection pressures. Different forms have developed for the same purpose, for example, SODs, peroxidases and GPx are important members of the antioxidant enzyme capacity group. Tocopherols and ascorbic acid, as representatives of the antioxidant network, are manufactured only in plants, but are needed by animals. Ascorbic acid is an essential antioxidant, but cannot be synthesized by *homo sapiens*. In humans, therefore, antioxidant defense against toxic oxygen intermediates comprises an intricate network which is heavily influenced by nutrition. GR, glutathione reductase; GSG, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; GST, glutathione-S-transferase; MSR, methionine sulphoxide reductase; PUFA, polyunsaturated fatty acids; S-AA, sulphur amino-acids; SH-proteins, sulphydryl proteins; SOD, superoxide dismutase; Fe Cu, transition metal-catalysed oxidant damage to biomolecules. Adapted from Strain [304] with permission from Cambridge University Press.

organic compounds that are not directly involved in normal growth, development and reproduction, but in long term survival and fecundity [302]. The plant secondary metabolites are largely involved in the chemical defense against herbivores, microbes, viruses and competing plants, in signaling and in nitrogen storage [303]; and some (e.g. polyphenols, carotenoids) also serve functions in the protection against ROS. The underlying metabolic pathways towards secondary metabolites lead to a series of related compounds that are usually composed of few major metabolites and several minor components differing in the position of their functional groups [303]. Animals consuming fruits and vegetables may employ these plant secondary metabolite networks for their own purposes, including maintenance of inflammatory/anti-inflammatory balance, cancer chemoprevention and protection against ROS [303].

In view of the yet poorly understood complex antioxidant networks composed of many compounds, it seems improbable to find a single 'magic bullet' to prevent and treat chronic diseases associated with ROS. Protective effects of fruits and vegetables may originate from their numerous phytochemicals working in concert [305] and from many different mechanisms of action that are not solely related to ROS. A purified phytochemical may not have the same health benefit as that phytochemical present in whole foods or a mixture of foods [250, 306]. In biological systems, toxins may become nutrients, while nutrients may become toxic in other situations [268], for example when disbalanced with other nutrients. Rather than translating our food into an assembly of nutrients where each has to prove its health benefits by scientific means, the objective should be to embrace a eucaloric diet that provides the adequate amount of nutrients from whole foods to maintain our body homeostasis. 'Adequacy' may in this sense be translated into causing an optimal interaction between our diet (and our lifestyle in general) with our genome, that is: nurture in balance with nature.

Evolutionary nutrition vs. randomized controlled trials

Coherence between lifestyle factors, including the composition of our diet, is quite obvious from an evolutionary point of view. After all, there was first an environment, and from this environment originated a genome that was adapted to that environment: it is the substrate (environment) that selects the organism, not *viceversa*. This is exactly what Darwin meant with 'conditions of existence', as the most important driving force in evolution. In other words, our only slowly changing genome is indissolubly linked to a certain environment and lifestyle. However, we have changed this environment since the agricultural revolution and continue to do so with still increasing paste. The resulting conflict does not generate acute toxicity, but acts as an assassin in the long term. Probably, the conflict does not exert much selection pressure either, because its associated mortality occurs mainly after reproductive age.

To solve the conflict, it is virtually impossible to study all of the introduced errors in our lifestyle (Table 1) in isolation, according to the reigning paradigm of EBM [307]. EBM is widely confused with the results of RCTs and preferably the meta-analysis thereof [308, 309]. This paradigm, originally designed for objective evaluation of medical treatments and drugs in particular, and named in nutrition research 'Evidence Based Nutrition' (EBN); is at present misused by food scientists and Health and Nutrition advisory boards. In contrast to drugs, this (expensive) RCT paradigm usually lends itself poorly for the study of single nutrients with meaningful outcomes [308]. For each nutrient, we are dealing with poorly researched dose-response relationships, multiple mechanisms of action, small effects causing pathology in the long-term, numerous interactions, ethical limitations regarding the choice of intervention and control groups, and the inability to patent its outcomes [309]. The RCT criteria are moreover inconsistently applied in the current development of nutritional recommendations. For example, there is no RCT-supported evidence for the saturated fat hypothesis [170], and also not for the *trans* fatty acids, while such an approach is considered mandatory for the adjustment of the vitamin D nutritional standards [310–312]. Incidentally, there was also no RCT prior to the introduction of *trans* fatty acids showing that they could be consumed without adverse effects on the long term. However, there is an RCT on the effects of smoking cessation, which showed an *equal* mortality among the quitters [313, 314]. The meta-analyses of RCTs studying the influence of LCP on brain development are negative [315–318]. However, recommendations for their addition to infant formulas have been issued [196], probably because nobody wants to take chances with the brains of our offspring. By applying EBM in a rigorous manner and by merely taking a view from the 'precautionary principle' (i.e. zero

risk [3]) this well meant concept has become a burden in the nutritional science, that calls for replacement by appropriate risk-cost-benefit analyses such as e.g. performed for vitamin D [319].

Our diet is composed of millions of substances that are part of a biological network. In fact, we eat 'biological systems' like a banana, a fish or a piece of meat. There is a connection between the various nutrients in these systems. In other words,, there is a balance and an interaction that is part of a living organism. This balance can be found in the reconstruction of our Paleolithic diet, and various attempts for this reconstruction have already been made [28, 131, 320–322]. Preliminary results of interventions with a Paleolithic diet are utterly positive (for a review see [323]). For example, in an indeed uncontrolled study with non-obese sedentary healthy subjects, an eucaloric Paleolithic diet resulted within 10 days in beneficial effects on three out of the four symptoms of the metabolic syndrome, i.e. blood pressure, dyslipidemia and glucose homeostasis. The fourth symptom, overweight/obesity, was deliberately not changed to prevent the attribution of any beneficial changes to weight loss [324].

Nurture, not nature

Less than 5% of our diseases can primarily be ascribed to heritable genetic factors [325, 326]. 'Genome wide association studies' (GWAS) will not make this figure change; not even if the number of patients and controls are further increased. As it could have been predicted from evolution, these GWAS identify only a few genes that are associated with typically Western diseases. Moreover, the so far identified genes merely convey low risks. In one of these disappointing GWAS, where 14,000 patients with seven major typically Western diseases and 3.000 controls were studied, it was concluded that: '... for any given trait, there will be few (if any) large effects, a handful of modest effects and a substantial number of genes generating small or very small increases in disease risk' [327]. The differences in genetic susceptibility to environmental factors is widely confused with a primary genetic origin of Western disease. Environmental factors may mimic genetic heritability, especially when the exposure has become widespread, As clearly explained by Rose [328]: "If everyone smoked 20 cigarettes a day, then clinical, case-control and cohort studies alike would lead us to conclude that lung cancer was a genetic disease; and in one sense that would be true, since if everyone is exposed to the necessary agent, then the distribution of cases is wholly determined by individual susceptibility". In other words: 'disease susceptibility genes' is a misnomer from an evolutionary point of view.

Most of the currently demonstrated polymorphisms associated with typically Western diseases already existed when *homo sapiens* emerged about 160,000 years ago in East-Africa. After all, the largest inter-individual genetic variation can be found between individuals belonging to a single population (93–95% of the total genetic variation), and only little genetic variation is on the account of differences between populations belonging to a single race (2%) and between the 5 races (3–5%) [329]. The allele that, according to current knowledge, is linked with the highest penetrance of type 2 diabetes mellitus in the general population (with Western lifestyle!) conveys 46% higher relative risk (RR=1.46) [330]. In contrast, a woman with a body mass index (BMI) of 35^{+} kg/m^2 has a one hundred-fold higher risk (RR=100) of diabetes mellitus type 2 [331], which translates into a 9,900% higher relative risk. 'Genetic' diseases with relative risks below 1.5 have no practical value in Public Health. They are only important to our understanding of the etiology of the concerning disease and for drug development [326], which is part of Health Care.

Between 70 and 90% of the cases of type 2 diabetes mellitus, CVD and colon cancer can be prevented by paying more attention to nutrition, smoking, overweight and lack of physical activity [325]. Hemminki et al. [326] stated that 'if the Western population was to live in the same conditions as the populations of developing countries, the risk of cancer would decrease by 90%, provided that viral infections and mycotoxin exposures could be avoided'. The popular counter argument that people in developing countries have (*on average!*) shorter life spans is not valid. The reason that we (*on average!)* live longer in Western societies, is mainly due to the strong reduction of infectious diseases (particularly in childhood), famine

3 The precautionary principle is a moral and political principle stating that, if an intervention or policy may cause serious or irreversible damage to society or the environment, the burden of proof lies with the proponents of the intervention or the measure if there is no scientific consensus on the future damage. The precautionary principle is particularly applicable in health care and environment; in both cases we deal with complex systems in which interventions result in unpredictable effects (source: Wikipedia).

and violence [332, 333], and also part on the account of Health Care. However, together with our increasing life expectancy, there is a decrease in the number of years without chronic disease [334].

Conclusions

It has become clear that most, if not all, typically Western chronic illnesses find their primary cause in an unhealthy lifestyle and that systemic low grade inflammation is a common denominator. From an evolutionary point of view, the current conflict between environment and our Paleolithic genome traces back to our brain growth and the ensuing intimate relationship between inflammation and metabolism. The present disbalance between inflammatory and anti-inflammatory stimuli does not originate from a single cause and can consequently also not be solved by a single 'magic bullet'. Resolution of the conflict between environment and our ancient genome might be the only effective manner to arrive at 'healthy aging' and to achieve this objective we might have to return to the lifestyle of the Paleolithic era according to the culture of the 21st century [16, 322].

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CHAPTER 1.2

Patients undergoing elective coronary artery bypass grafting (CABG) exhibit poor pre-operative intakes of fruit, vegetables, dietary fiber, fish and vitamin D

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Abstract

Coronary heart disease (CHD) may ensue from chronic systemic low-grade inflammation. Diet is a modifiable risk factor for both and its optimization may reduce post-operative mortality, atrial fibrillation and cognitive decline. We investigated the usual dietary intakes of patients undergoing elective coronary artery bypass grafting (CABG), emphasizing on food groups and nutrients with putative roles in the inflammatory/anti-inflammatory balance. From November 2012 to April 2013 we approached 93 consecutive patients (80% men) undergoing elective CABG. Of these, 55 were finally included (84% men, median 69 years, range: 46–84). Median BMI was 27 (range: 18–36) kg/m^2. Intakes (median; range) were: fruits (181; 0–433 g/day), vegetables (115; 0–303 g/day), dietary fiber (22; 9–45 g/day), EPA+DHA (0.14; 0.01–1.06 g/day), vitamin D (4.9; 1.9–11.2 µg/day), saturated fat (13.1; 9–23 energy%) and linoleic acid (6.3; 1.9–11.3 energy%). The percentages of patients with intakes below recommendations were: 62% (fruits; recommendation: 200 g/day), 87% (vegetables; recommendation: 150–200 g/day), 73% (dietary fiber; recommendation: 30–45 g/day), 91% (EPA+DHA; recommendation: 0.45 g/day), 98% (vitamin D; recommendation: 10–20 µg/day) and 13% (linoleic acid; recommendation: 5–10 energy%). Percentages above recommendations were: 95% (saturated fat; recommendation: <10 energy%) and 7% (linoleic acid). Dietary intakes of patients proved comparable with the average nutritional intake of the age- and sex-matched healthy Dutch population. These unbalanced pre-operative diets may put them at risk of unfavourable surgical outcomes, since they promote a pro-inflammatory state. We conclude that there is an urgent need for intervention trials aiming at rapid improvement of their diets to reduce peri-operative risks.

Keywords

CABG surgery, coronary artery disease, diet, low-grade inflammation

Abbreviations

AHA, American Heart Association; CABG, coronary artery bypass grafting; CHO, carbohydrates; CHD, coronary heart disease; DNL, *de novo* lipogenesis; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; HES, healthy eating score; LA, linoleic acid; Nrf2, nuclear factor erythroid-2 related factor; PUFA, polyunsaturated fatty acids; RDA, recommended dietary allowance; RCT, randomized controlled trial; SFA, saturated fatty acids.

Conflict of Interest and Funding Disclosure

No conflicts of interest

Introduction

Atherosclerosis is a major cause of coronary heart disease (CHD) and its pathophysiological cascade is increasingly recognized to ensue from lifestyle-induced chronic systemic low-grade inflammation [1]. Modifiable risk factors for CHD are, among others, diet, smoking, hypertension, diabetes, and lipid disorders [2]. Coronary artery bypass grafting (CABG) is the standard revascularization treatment for patients with severe atherosclerotic heart disease, such as left main and three-vessel CHD [3]. CABG is a major event that unavoidably induces an inflammatory response, mostly on top of an already existing state of systemic low-grade inflammation. The relatively old age of patients undergoing CABG, the often widespread atherosclerotic disease and the presence of CHD risk factors, confer additional risks for vascular sequels [4], frequently coinciding with (most often transient) paucity in brain function, the so-called post-operative cognitive dysfunction [5].

An inflammatory reaction is essential for survival from various events. It is the reflection of an activated immune system aiming to protect us from traumas, invading pathogens or a response to sterile infection [6]. The systemic inflammatory response syndrome (SIRS) has become a widely accepted concept to explain the pathophysiology of systemic inflammation from multiple aetiologies, i.e. trauma, surgery and infections [7]. However, uncontrolled and secondary reactions resulting from an activated immune system harbour the capacity to kill us and therefore, any inflammatory reaction that has started should subsequently be ended, in order to limit the unavoidably associated collateral damage [8]. Anti-inflammatory responses compensating for the SIRS have been named the counter anti-inflammatory response syndrome (CARS) [9], characterized by a weakened host defence and augmented susceptibility to secondary infections. Consequently, restoration of an injured host to homeostasis requires the simultaneous involvement of both the SIRS and the CARS, while any disruption of their balance towards the extremes may result in, for example, immune suppression, cardiovascular collapse and organ failure [9]. In line with this view, it is plausible that, in some susceptible patients, CABG surgery acts as the catalyst for complications that were not present pre-operatively; while in others, it constitutes a burden on top of a system already showing decline [10].

There are numerous false inflammatory triggers in our current, typically Western, lifestyle that may collectively lead to a state of chronic systemic low-grade inflammation, eventually leading to atherosclerosis and CHD. These false triggers hijack an evolutionary conserved interaction between our immune system and metabolism [11]. Among the pro-inflammatory factors in our current diet, we find, the consumption of *trans* fatty acids, a high ω6/ω3 fatty acid ratio, a low intake of ω3 fatty acids from fish, a low vitamin D status, a low-fiber diet, and low intakes of fruit and vegetables [12]. Nevertheless, less than 25% of the Dutch population follows the recommendations regarding the consumption of fruit, vegetables and dietary fiber, and in 1998, the average fish consumption in The Netherlands amounted to hardly 3 times per month [13]. Eating fish once a week has been associated with a 15% lower risk of CHD death compared with a consumption of less than once per month [14].

The aim of the present study was the assessment of the dietary intake in patients undergoing elective CABG, with emphasis on food groups and nutrients known to play important roles in the inflammatory/anti-inflammatory balance. Habitual dietary intakes were estimated by means of a food frequency questionnaire (FFQ). We anticipate that the results will enable us to identify (subgroups of) patients with high risk of (post)-surgical complications with the ultimate aim to develop strategies for minimizing post-CABG complications and their ensuing comorbidities by targeting diet as a relatively-easy-to-modify risk factor.

Experimental methods

Study design and study group

Patients were recruited from two cardiothoracic nursing units of the Universal Medical Center Groningen (UMCG), The Netherlands. Between November 2012 and April 2013, all consecutive patients admitted for elective CABG surgery were approached for voluntary participation (93 patients; 74 males, 19 females). Of these, 16 (17%) declined participation due to the extent of the FFQ (n=8, 50%), not being able to read (n=4, 25%) or understand and/or capable to read Dutch (n=1, 6%), confusion (n=1, 6%), or being too occupied with diagnostic examinations (n=2, 12%). Of the remaining 77 who agreed to fill out the FFQ, 55 were ultimately included (71%; 46 males, 9 females). Reasons for dropping-out were: incomplete questionnaires (n=10, 13%), CABG surgery not being performed (n=5, 6%), incorrectly filled out questionnaires (n=3, 4%) and others [1 patient not scheduled

Table. Food frequency questionnaire outcomes for 55 patients awaiting CABG, as compared with dietary recommendations for the Dutch and Americans

		Patients	Dutch recommendations			USA recommendations		
	Unit	Median (range)	Guideline	% (n) below	% (n) above	Guideline	% (n) below	% (n) above
Energy	(kcal/day)	2,225 (1,091–4,433)	Female ≤50 y: 2,317[1]	0 (0)	100 (1)	Female ≤ 50 y: 1800[4]	0 (0)	100 (1)
			Female 51-70 y: 2,150[1]	75 (3)	25 (1)	Female > 51 y: 1600[4]	63 (5)	37 (3)
			Female >70 y: 1,863[1]	25 (2)	25 (2)			
			Male ≤50 y: 2,914[1]	0 (0)	100 (1)			
			Male 51-70 y: 2,627[1]	30 (8)	70 (19)	Male ≤ 60 y:2200[4]	13 (1)	87 (7)
			Male >70 y: 2,221[1]	44 (8)	56 (10)	Male > 60 y: 2000[4]	39 (15)	61 (23)
Fruit	(g/day)	181 (0–433)	200[2]	62 (34)	38 (21)	4-5 servings (200–250 g)[4]	62 (34)	22 (12)
Vegetables	(g/day)	115 (0–303)	200[2]	87 (48)	13 (7)	4-5 servings (200–300 g)[4]	87 (48)	2 (1)
Dietary fiber	(g/day)	22 (9–45)	25[2]	73 (40)	27 (15)	Female: 22[4]	78 (7)	22 (2)
						Male: 28[4]	78 (36)	22 (10)
Carbohydrates	(en%)	44 (24–58)	40–70[3]	27 (15)	0 (0)	45–65[5]	58 (32)	0 (0)
Protein	(en%)	15 (9–21)	10–25[1]	2 (1)	0 (0)	10–35[5]	2 (1)	0 (0)
Fat	(en%)	35 (26–51)	20–40[1]	0 (0)	22 (12)	20–35[5]	0 (0)	49 (27)
Saturated fat	(en%)	13 (9–23)	Upper limit: 10[2]	5 (3)	95 (52)	<10[4]	5 (3)	95 (52)
						<7[5]	0 (0)	100 (55)
Trans fat	(en%)	0.6 (0.3–1.0)	Upper limit: 1[2]	98 (54)	2 (1)	<1[6]	98 (54)	2 (1)
LA	(en%)	6.3 (1.9–11.3)*	At least 2[3]	2 (1)	98 (54)	5–10[7]	13 (7)	7 (4)
EPA + DHA	(g/day)	0.14 (0.01–1.06)*	0.45[3]	91 (50)	9 (5)	1[5]	98 (54)	2 (1)
Vitamin D	(µg/day)	4.9 (1.9–11.2)*	<70 y: 10[3]	97 (28)	3 (1)	<70 y: 15[5]	100 (29)	0 (0)
			≥70 y: 20[3]	100 (26)	0 (0)	≥70 y: 20[5]	100 (26)	0 (0)

Outcomes of the study group are medians (range)

* Not corrected for supplement intake (dosage not known)

[1] Data from [22]; [2] Data from [16]; [3] Data from [21]; [4] Data from [23]; [5] Data from [25]; [6] Data from [86]; [7] Data from [87].

Abbreviations: DHA, docosahexaenoic acid; en%, energy percent; EPA, eicosapentaenoic acid; LA, linoleic acid.

for CABG (1%); 2 patients not returning the FFQ (2%) and 1 death (1%)].

FOOD FREQUENCY QUESTIONNAIRE

For the estimation of nutritional intakes we used a FFQ from the Division of Human Nutrition of Wageningen University, The Netherlands. This questionnaire is based on the FFQ developed and validated by Feunekes et al. for fats and cholesterol [15], and was updated with the Dutch National Food Consumption Survey 2007–2010 [16] and the Dutch National Food Composition Database 2010 v.2.0 [17]. The composed FFQ was evaluated using the mean of three 24-hour recalls as the reference method [18], where fair associations were encountered for vitamin D, dietary fiber, vegetables and fruit [18]. For the same FFQ, very strong associations were found for energy intake [19]. Patients were asked to report on their dietary habits during the previous four weeks. In case they had changed their diets because of sickness or followed a special hospital diet within this period, they were requested to provide information of the last four weeks that they ate according to their regular dietary habits. The completeness of the information was checked by a trained researcher using a specific checklist. Correctly filled out FFQs were digitized and processed using the *Dutch FFQ-TOOLTM*, which is a computer system that generates and processes FFQs tailored for specific research questions and/or populations [20]. In the *Dutch FFQ-TOOLTM*, the nutrient composition of each food item is derived from the weighted mean composition of the single foods of which the food item consists [20], as weighted by the reported amounts in the Dutch Food Composition Database 2010 [17].

Data on age of the subjects were obtained by interviews in the Dutch language. Weight and height were self-reported or measured on the spot. The participants were asked whether they had gained or lost weight in the past year and whether they consumed vitamin or mineral supplements, i.e. vitamin D, (multi)vitamins and/or fish oil.

Data analysis

Outcome data of the FFQ were compared with the Dutch [16, 21, 22] and American [23, 24] dietary recommendations. Because of their physical condition, we considered the patients as sedentary, and compared their energy intakes with those recommended for the equivalent Physical Activity Level for each group. We visually compared the outcomes with the dietary intakes of representative age- and sex-matched healthy subjects in the Dutch general population. For this comparison, we calculated the 5th, 50th and 95th percentiles for the outcomes of the male and female patients, and related them with the corresponding 5th, 50th and 95th percentiles of the healthy population, as established in the Dutch Food Consumption Survey 2007–2010 [16].

Healthy Eating Score and adherence to recommendations

A "healthy eating score" (HES) was calculated from four items of main interest, i.e., the intakes of fruits, vegetables, fiber and EPA+DHA. For each of these items, patients were categorized according to their quintiles of intake to obtain a score from 1 (lowest quintile) up to 5 (highest quintile). The outcomes of the four items were summed, obtaining the HES, in which the minimum score was 4, while 20 constituted the maximum. We also investigated whether individual patients adhered to the recommendations for each of these four nutrients/food items. These numbers (0–4) were plotted in a frequency curve showing how often the sums of these 4 recommendations were fulfilled.

Statistics

Percentiles and graphs for visual comparison and for HES calculation were performed with PASW version 18.0 (SPSS Inc, Chicago, IL).

Results

Characteristics of the study population

Fifty-five patients (46 males, 9 females) with a median age of 69 years (range 46–84) were included. Median height was 176 cm (range 160–204), median weight 83 kg (range 47–120) and median body mass index 27 kg/m^2 (range 18–36). Within the whole study group, eight patients reported taking some form of supplement. Three of them took a vitamin D supplement, three a vitamin C supplement, two a multivitamin, and one a supplement containing both ω3 and ω6 fatty acids. Dosages were not specified and therefore, not used in the calculations.

Outcome of food frequency questionnaire compared to Dutch and USA recommendations

The Table shows the outcome of the FFQ and its comparison with dietary recommendations for the Dutch [16, 21, 22] and Americans [23, 24].

Energy intake

The median energy intake in our study group was 2,225 kcal (range 1,091–4,433). Twenty-one patients (38%) reported energy intakes below the Dutch and American requirements for their specific age and sex groups (Table), while 34 patients (62%) presented energy intakes above these requirements.

Fruit, vegetables and dietary fiber

The median fruit intake of our study group was 181 g/day (range 0–433) (Table), while the Dutch recommended fruit intake is 200 g/day, and the American guideline amounts to 200–250 g/day (4–5 servings/day). In our study, 62% of the patients reported a fruit intake below, and only 38 and 22% reported fruit intakes equal or above the respective Dutch and American recommendations.

The median vegetable intake of our group was 115 g/day (range 0–303). The recommended vegetable intake in The Netherlands is 200 g/day, and 200–300 g/day (4–5 servings/day) in the USA. Pursuant to these quantities, 87% of the patients failed to fulfil the Dutch or the American recommendations, while only 13 and 2%, respectively, did fulfil them. Fiber consumption was below recommendations (25 g/day in The Netherlands and 22–28 g/day in the USA) in 73 and 78% of the patients (median 22, range 9–45 g/day), and 27 and 22% of them reported a fiber

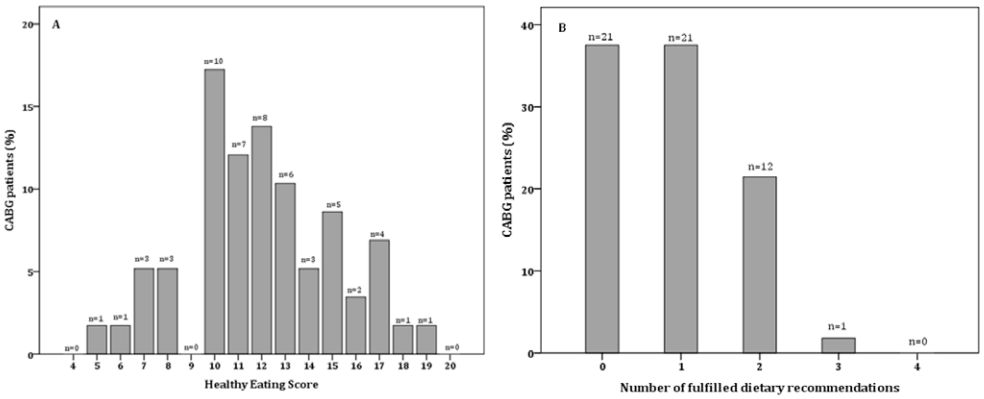

Figure 1. "Healthy eating score" and degree of dietary recommendation-fulfilment for 55 patients awaiting CABG

Panel A shows the percentages of patients with indicated Healthy Eating Scores (HES) ranging from 4-20. HES was obtained by dividing four important dietary variables into quintiles. These were the intakes of vegetables, fruit, dietary fiber and EPA+DHA. For each variable, patients were given a score, from 1 (lowest quintile) to 5 (highest). For each patient, the scores were summed to obtain the HES (minimum 4; maximum 20). There seems to be an arbitrary subgroup with scores of 8 and below. Panel B shows the distribution of the number of recommendations that the various patients fulfilled. It was found that 76% (n=42) of the patients adhered to none of the four recommendations or just one of them. None of the patients adhered to all 4 recommendations.

Abbreviations: CABG, coronary artery bypass grafting.

consumption equal or above these recommendations, respectively.

Carbohydrates, protein and fat

The median energy intake from carbohydrates (CHO) in our patients was 44 energy% (range 24–58) (Table). The Dutch and American recommendations are 40–70 and 45–65 energy%, respectively. Consequently, 27% of the patients reported a CHO intake below the Dutch recommendation and 58% below the USA recommendation, while none of the patients reported CHO intakes equal or beyond these recommendations. Protein intake was below the recommendations in only 2% of the study population (median intake 15 energy%, range 9–21; recommendations 10–25 energy% in The Netherlands and 10–35 energy% in the USA). The median fat consumption was 35 energy% (range 26–51), which is within the range of both the Dutch (20–40 energy%) and American (20–35 energy%) recommendations. None of the patients reported a fat intake below these recommendations, but 22 and 49% of the study group reported fat intakes equal or above the Dutch and American recommendations, respectively.

Saturated fat, trans fat, linoleic acid, EPA and DHA

The median consumption of SFA (13 energy%, range 9–23) (Table) was above the upper limit of the Dutch and American recommendations (both 10 energy%). None of the patients reported a SFA intake below the American Heart Association (AHA) recommendation of <7 energy% [24].

Trans fat consumption (median 0.6 energy%; range 0.3–1.0) was below the upper limit of 1 energy% recommended in The Netherlands and by the AHA [24] in 98% of the patients (all except for 1 patient; 2%).

The Dutch linoleic acid (LA) recommendation of *at least* 2 energy% to prevent essential fatty acid deficiency [21] was fulfilled by 98% of the study population (median 6.3; range 1.9–11.3 energy%). LA consumption was within the American recommendations (acceptable macronutrient distribution range: 5–10 energy%) in 80% of our study group, while 13% reported intakes below and 7% intakes equal or above the recommended range.

The consumption of EPA+DHA (median 0.14 g/day; range 0.01–1.06) was below the Dutch recommendations of 0.45 g/day in 91% of the patients. Except for one patient (2%) (who was not the patient taking ω3 and ω6 fatty acid supplements), none of them

complied with the AHA recommendation of 1 g/day for patients with documented CHD [25].

Vitamin D

The recommended dietary allowance (RDA) for vitamin D in The Netherlands is 10 µg/day (0–69 years) and 20 µg/day (≥70 years). Irrespective of sunlight exposure, all 0 to 4-year-old children, pregnant women and 50 to 70-year-old women are advised to take a supplement of 10 µg/day, while all subjects ≥70 years are recommended to take a supplement of 20 µg/day. Females (4–50 years) and males (4–70 years) are recommended to take a 10 µg/day supplement if they are insufficiently exposed to vitamin D-producing-sunlight. The latter is applicable for subjects living in The Netherlands from November until February [26] and greatly overlaps the current study period. This implies that all patients <70 years should be taking 10 µg vitamin D/day, while all ≥70-year-old patients should be taking 20 µg/day. Except for one patient (3% of the study population below 70 years, 2% of the total study population), none of them reported a vitamin D intake from food (median 4.9 µg/day, range 1.9–11.2) that reached the daily needs, while none of them complied with the American recommendations (for unexposed subjects: 15 µg/day for 1–69 years, and 20 µg/day for ≥70 years). Except for three patients (5%; dosage unknown), none of them reported the use of a vitamin D supplement.

Healthy eating score and percentage fulfilment of recommendations

The results for the calculation of the HES are shown in Figure 1 (panel A). None of the patients reached the minimum and maximum scores of 4 and 20, respectively. The distribution showed an arbitrary subgroup of 8 patients (15%) with scores of 8 and below. The distribution of the number of fulfilled dietary recommendations (Figure 1, panel B) showed a steep decrease from 38% of patients (n=21) who did not fulfil any of the four recommendations and 38% fulfilling only one of the recommendations, to zero patients fulfilling all four recommendations.

Food frequency questionnaire outcome compared with the healthy Dutch population

Figure 2 shows the dietary intakes of the CABG patients compared with those of their age- and sex-matched healthy counterparts in The Netherlands. Visual inspection revealed that the median fruit consumptions by the male and female patients appeared somewhat higher than the corresponding intakes by their age- and sex-matched healthy counterparts (Figure 2, panel A). For the female patients, the median fruit consumption was just above the Dutch and American recommendation of 200 g/day, but this was not the case for healthy females and the two male groups, who presented lower intakes. Of the healthy Dutch men, at least 75% did not adhere to the recommended fruit intake.

For vegetables (Figure 2, panel B), the median consumption by female patients was similar to that of their age-matched healthy Dutch counterparts, whereas in male patients, the median intake appeared lower. In all groups, approximately 75% showed vegetable intakes that did not meet the recommendations.

Median dietary fiber consumption by female patients was similar to the median consumption by their healthy Dutch counterparts (Figure 2, panel C). Both groups presented median fiber intakes below the Dutch and American recommendations. The median fiber intake by male patients was somewhat higher than their healthy counterparts and was similar to the Dutch recommendation.

Median EPA+DHA consumptions of both the female patient and healthy groups were similar, but the consumption of EPA+DHA of the male patients seemed somewhat higher than that of their age-and-sex-matched healthy counterparts (Figure 2, panel D). In all four groups, at least 75% of the intakes did not reach the Dutch recommendations and all EPA+DHA intakes were far below the AHA recommendation for subjects with documented cardiovascular disease [25].

Discussion

We estimated the dietary intakes of patients undergoing elective CABG using a FFQ. To the best of our knowledge, such data have not been reported before, with the exception of energy intakes [27]. A recent study in Iranian CHD patients [28] reported a low fish consumption within this group, together with a fruit and vegetable consumption within the 'safe' recommended range. Our main focus was set on food groups and nutrients with putative key roles in the inflammatory/anti-inflammatory balance. Our study population was relatively old (69 years; range 46–84) and predominantly overweight/obese (27 kg/m^2; range 18–36). The majority of the patients did not reach the

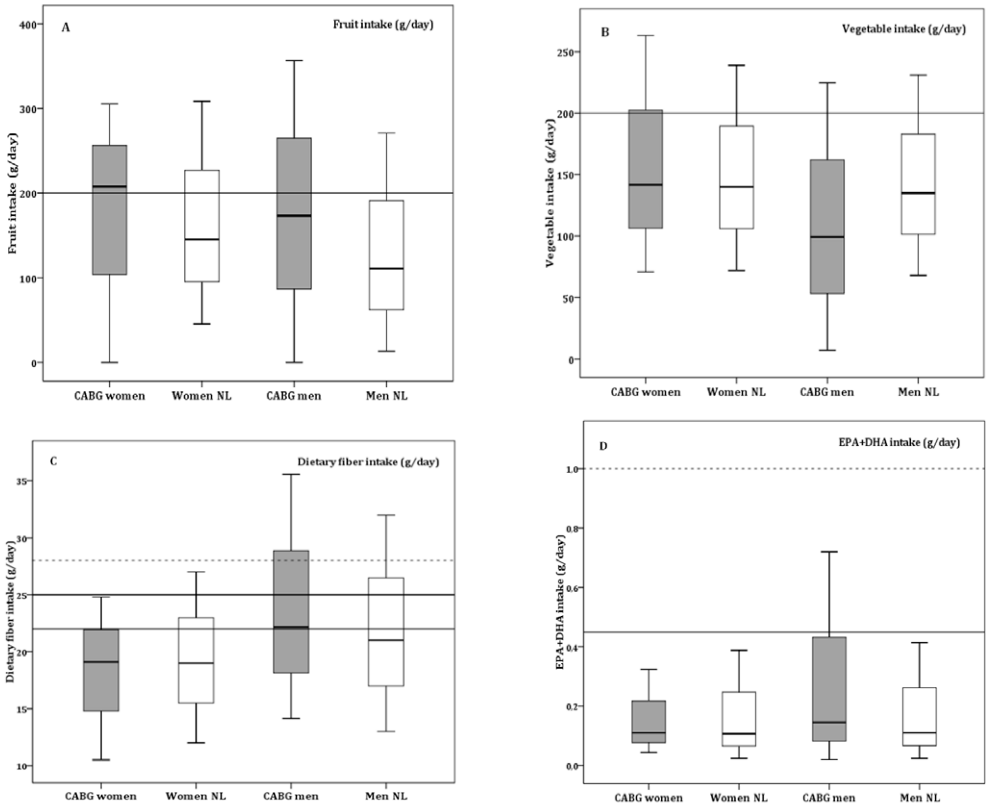

Figure 2. Outcome of the food frequency questionnaire for 55 patients awaiting CABG, as compared with data from healthy counterparts participating in the Dutch Food Consumption Survey 2007–2010

Data are presented as boxplots for the estimated intakes of fruits (panel A), vegetables (panel B), fiber (panel C) and EPA+DHA (panel D). Full lines represent the Dutch and the USA recommendations for fruit and vegetables (panels A and B), the USA recommendations for fiber for females (panel C), and the Dutch recommendations for EPA+DHA (panel D). Dotted lines represent the USA recommendation for fiber for males (panel C) and the AHA recommendation for patients with established CHD (panel D). Bold line represents the Dutch recommendation for fiber (panel C).

Abbreviations: CABG, coronary artery bypass grafting; CABG men, male patients awaiting CABG; CABG women, female patients awaiting CABG; Men NL, age-matched males from the Dutch Food Consumption Survey 2007-2010; NL, The Netherlands; Women NL, age-matched females from the Dutch Food Consumption Survey 2007-2010.

recommended intakes of vegetables (87%), fruits (62%), fiber (73%), EPA+DHA (91%) and vitamin D (98%), while 95% of them did not adhere to the recommendation to take a vitamin D supplement. There were no appreciable differences between the above intakes by our patient group and their healthy age- and sex-matched counterparts in the general Dutch population.

Systemic low-grade inflammation is considered to play a central role in many, if not all, typically Western diseases [12]. It is even likely to be a causal factor in the pathophysiological cascade towards CHD [1]. Diets low in ω3 fatty acids, natural antioxidants, fiber, and vegetables and fruits, have been associated with low-grade inflammation [29], and the same applies for a low vitamin D status [30]. We will therefore discuss our findings in the context of low-grade inflammation, CHD and post- CABG cognitive dysfunction [5], the latter being a major complication after surgery in the framework of brain inflammatory diseases.

Long chain ω3-polyunsaturated fatty acids

The majority of the patients (91%) did not reach the recommended intakes of fish EPA+DHA. Numerous studies support the important role of dietary fatty acids in low-grade inflammation. The metabolites of arachidonic acid, EPA and DHA are intimately involved in wound healing and other immune challenges [31]. The initial GISSI-Prevenzione [32] and the JELIS studies [33] supported the use of fish oil supplements in secondary prevention of CHD, but a recent meta-analysis [34] and systematic review [35] yielded conflicting results, probably because fish oil may not add a preventive effect on top of the highly effective current drug treatment [36]. However, EPA and DHA also exhibit protective effects on cognitive decline and depression [37], conditions that increasingly become recognized as inflammatory diseases [38, 39]. In rats, brain function is sensitive to dietary ω3-PUFA intake, since ω3-PUFA deprivation during 15–18 weeks reduced brain DHA levels and induced functional brain changes, increasing aggression and depression scores [40].

Saturated fatty acids, linoleic acid and trans fatty acids

Almost the entire group (95%) reported a SFA intake above the recommendations, 98% reported a LA intake above the recommended intake of *at least* 2 energy% and only 2% reported a *trans* fatty acid intake above the recommendations. The widely accepted harmful effects of dietary SFA in CHD are increasingly questioned [41], while in recent meta-analyses of RCTs, it was concluded that partial replacement of dietary SFA for LA, the dominating dietary PUFA, insignificantly increases CHD risk [42]. Furthermore, SFA intake alone is not a predictor of CHD, whereas the intake of industrially produced *trans* fatty acids is associated with CHD, independent of other dietary and CHD risk factors [43]. Despite the prominent contributions of dairy products and meat to SFA intake, neither of them have been consistently associated with CHD risk [44, 45].

There are many factors interacting with dietary SFA, and the outcome of these interactions may determine whether SFA will accumulate in the body, will be *de novo* synthesized, and will eventually contribute to low-grade inflammation [46]. An important factor is a high CHO intake (further discussed), which promotes *de novo* lipogenesis (DNL), including *de novo* SFA synthesis, and, in addition, the sparing of dietary SFA [47].

Carbohydrates

CHO intake was within the recommended intake in 73% of our study population. High-CHO low-fat diets and insulin resistance have been shown to augment DNL [48], and thereupon, to induce the synthesis of pro-inflammatory SFA, notably palmitate. CHD patients are likely to present low insulin sensitivity [49], secondary to lifestyle-induced systemic low-grade inflammation [12]. This condition might be at the basis of the CHD aetiology, and become aggravated by the sedentary lifestyle that usually comes along with the progression of disabling CHD. Taken together, this implies that high-CHO (especially CHO with a high GL/GI) high-SFA diets are contraindicated in this patient group.

Dietary fiber

The majority of our study group (73%) reported a fiber intake below the dietary recommendations. The relationship between fiber and low-grade inflammation is complex. A high fiber diet may decrease the ratio between the Gram positive Firmicutes and the Gram negative Bacteroidetes in the gut [50]. This shift coincides with the colonic fermentation of fiber by flora selection [51], stimulates short-chain fatty acid production [50], may improve gut integrity [52] and thereby, may reduce chronic inflammation. In the same scenario, by virtue of their interchangeability at constant CHO-intake, the often quoted anti-inflammatory capability of a high-fiber intake proved less consistent than for diets with low glycemic indices (GI) or glycemic loads (GL) [53]. These data suggest that high dietary fiber might be a proxy for a low-GI/GL diet, and highlights the pro-inflammatory effect of a high GI/GL [12].

Vegetables and fruit

Only 13% and 38% of our study group reported a vegetable and fruit intake within the dietary recommendations, respectively. Diets low in vegetables and fruit are associated with inflammation [12] and are also convincingly associated with hypertension, CHD and stroke [54]. Meta-analyses of prospective studies indicate that less-than-3 vs. 5+ servings of vegetables and fruit per day correspond with a 17% CHD reduction [55]. A recent study reported a robust inverse relation of vegetables and fruit consumption with cancer, CHD and all-cause mortality, with

benefits up to 7+ portions per day [56]. Vegetables and fruit have the highest (micro)nutrient density scores of all major food groups [57]. They are not only rich in protein, fiber, vitamins and minerals, but also in antioxidants, which may collectively be responsible for their protective effects on chronic diseases, including CHD [58]. Plants also contain 'secondary metabolites', which increase the plants' overall ability to survive and overcome local challenges [59]. Animals (humans included) consuming vegetables and fruits may employ these networks of phytochemicals for their own purpose, including maintenance of the inflammatory/anti-inflammatory balance [60] and brain function enhancement [59]. Some of these, such as curcumin from the *Curcuma longa* plant, and sulforaphane from cruciferous vegetables (e.g. broccoli), activate nuclear factor erythroid-2 related factor (Nrf2), a key regulating transcription factor of inducible defence systems in the body [61]. Nrf2 regulates about 200 genes by binding to their antioxidant response elements [62], causing the coordinated expression of proteins involved in the inhibition of inflammation, detoxification, antioxidant systems, activation of other transcription factors, and the metabolism of lipids, CHO, nucleotides and amino acids [63]. Insufficient Nrf2 activation has been linked, among others, to CHD [64, 65] and brain degenerative diseases, such as Parkinson's and Alzheimer's disease [66].

Vitamin D

We found that 98% of our study group reported insufficient vitamin D intake, while 95% did not adhere to the recommendation to take a vitamin D supplement. As a modulator of inflammatory cells and inflammatory cytokines secretion, low vitamin D status may contribute to chronic inflammatory conditions [67]. Several epidemiological studies have linked inadequate vitamin D status to higher susceptibility of immune-mediated disorders [68], including CHD [30]. Vitamin D deficiency affects cardiac contractility, vascular tone, cardiac collagen content, and cardiac tissue maturation, and has direct effects on vascular smooth muscle cell calcification and proliferation [69]. A recent study has shown a high prevalence of vitamin D deficiency in cardiac surgical patients, associated with a two-fold higher risk of major adverse cardiac and cerebrovascular events after surgery [70]. This raises the question of whether vitamin D supplementation before surgery may reduce the risk of adverse events. In line with these data, a meta-analysis of prospective cohort studies demonstrated an inverse association between circulating 25(OH) vitamin D levels (ranging from 20 to 60 nmol/L) and the CHD risk [71]. In addition, there is a growing body of research exploring the association between vitamin D levels with diverse adult neuropsychiatric diseases [72].

Potential effect of diet on CABG surgery outcome

CABG surgery may be a trigger of an exaggerated inflammatory reaction, resulting in vascular sequels, particularly those leading to neurological dysfunction [4]. In this context, correction of nutrient status might be more effective in primary and secondary prevention than in the treatment of an acute event [73]. The aim should therefore be set in prevention. A healthy food pattern has indeed been identified as a factor that supports health, lowers BMI, and could potentially promote better operative outcomes in terms of quality of life for at least patients with the metabolic syndrome and cardiac problems [74]. This concept appears, however, to be based on the reduction of certain dietary elements (e.g. saturated fat, added sugars, and sodium), rather than on the benefits of other nutrients that are part of a healthy diet [57]. Moreover, modest dietary changes harbour low risk, are relatively inexpensive, and are widely available compared with drugs, invasive procedures, and devices [75].

The above raises the question whether a rapid correction of nutrient status is possible, prior to the CABG intervention. Some nutrients, such as water soluble vitamins, may be rapidly corrected, but others, e.g. fat soluble nutrients, might be less readily distributed among key organs, such as the brain. As an example, DHA half-life in the human brain amounts to approximately 2.5 years [76], which might be the reason why intervention trials with fish oil in neurological diseases, e.g. Alzheimer's, show conflicting result [77, 78]. On the other hand, short-term consumption (2 weeks) of a diet high in fruit and vegetables (12 servings/day) induced significantly lower lymphocyte DNA damage and pro-inflammatory cytokine production in overweight women compared with an isocaloric diet low in fruit and vegetables (2 servings/day) [79]. In addition, only 10 days [80] or two weeks [81] consumption of an isocaloric Palaeolithic type diet, comprising lean meat, fruits, vegetables and nuts, and excluding cereal grains, dairy or legumes, has been demonstrated to improve blood pressure and glucose tolerance, decrease insulin secretion, increase insulin sensitivity

and improve lipid profiles without [80] or with minimal [81] weight loss in healthy sedentary humans [80] and subjects with at least 2 characteristics of the metabolic syndrome [81].

Limitations

Our study has many limitations. The participants derived from a single hospital and current results might therefore not be generalizable across The Netherlands. However, the similarity of their diets with their counterparts in the healthy Dutch population does not argue in favour of this notion. On the contrary, the employed drop-out criteria rather suggest that the current study group is within the 'better nourished' share of the target population and that our results may even be considered 'overoptimistic'. Food pattern was determined by a self-administered questionnaire based on the memory of the participants. The questionnaires used by the Dutch Health Council and the FFQ used with our study group may not produce the exact same outcomes. However, both questionnaires were translated into the intakes of certain nutrients and then validated, enabling both outcomes to be comparable. Nevertheless, FFQs are prone to significant systematic and random errors, which can unfavourably affect their analysis and interpretation [82]. One of the most common biases is the underreporting of daily energy intake [19, 27] and unhealthy food [82] by people with high BMI. We did not collect data on the intake of salt, or other CHD associated lifestyle factors, such as lack of physical activity, insufficient sleep, chronic stress and smoking [83]. We also did not investigate vitamin B12. Vitamin B12 deficiency is a common cause of neuropsychiatric symptoms in the elderly [84]. These factors are interrelated and may therefore either ameliorate or aggravate the presently encountered poor diet.

Conclusions

We conclude that our study population presents poor dietary habits which may theoretically put them at risk of a pro-inflammatory state that may worsen their disease and unfavourably affect surgery outcome. There is an urgent need for intervention trials and education policies aiming at rapid improvement of their unbalanced pre-operative diets to reduce the risk of postoperative death or complications, such as atrial fibrillation and cognitive decline. Dependent on body weight, these interventions should, in our minds, aim at iso- or hypocaloric diets, with moderate-CHO (e.g. 40 energy%), moderate-high protein (e.g. 25 energy%), moderate-fat (35 energy%) and supplementation with vitamins D and B12. This translates into a low-GL, fiber-rich diet that is abundant in micronutrients and phytochemicals from vegetables, fruits and nuts, together with lean meat and (EPA+DHA)-rich fish [85].

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Conflict of interest

None

Authorship

B., M.A., M.J.L., D.A.J. and F.A.J. designed research; B. and G.H.A.M. conducted research; B.,G.H.A.M. and J.H.M. analyzed data; B., and F.A.J. wrote the paper. F.A.J. had primary responsibility for final content. All authors read and approved the final manuscript.

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CHAPTER 1.3

To Restore Health, "Do we Have to Go Back to the Future?" The Impact of a 4-Day Paleolithic Lifestyle Change on Human Metabolism — a Pilot Study

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Abstract

On their way from the Stone Age via the Agricultural Revolution to current high-tech conditions, humans lost their primal foraging behavior. Today, energy expenditure is not necessary anymore for gathering nor hunting, and metabolic diseases are epidemically arising wherever our original Paleolithic lifestyle is turning into a modern sedentary lifestyle. In this pilot study, we followed through the concept that a radical change towards a Paleolithic hunter-gatherer lifestyle could serve as therapy against any metaflammatory disease, even in the short term. Thirteen healthy adult volunteers were transferred to the DELUX National Park (Germany and Luxembourg) for four days and three nights, where Stone Age conditions where mimicked. Thirty eight biochemical and bioelectrical parameters were measured from participants before and after this relocation. Body weight (–3.9%), body fat (–7.5%), body mass index (-3.8%), visceral fat area (–14.4%) and metaflammation-related parameters (fasting glucose = –18.2%; fasting insulin = –50.1%; HOMA = –57.8%) decreased significantly. C-reactive protein, as the main indicator for low-grade inflammation, increased up to an average of 169.6%. Our data show that returning to our Paleolithic roots may have positive effects on risk factors commonly associated with metabolic disorders, such as obesity and type 2 diabetes. These findings may lead the way to further research to answer the question whether the already existing metabolic conditions and/or autoimmune and neuroinflammatory diseases could be influenced by a Paleolithic lifestyle.

Keywords

Paleo diet, Metabolic syndrome, Low-grade-inflammation, Metainflammation, HOMA-index, Sedantary lifestyle

Background

According to current data from the World Health Organization (WHO), for the first time in human history, more people suffer from overweight than undernutrition. Worldwide, the prevalence of obesity has doubled since 1980 [1]. It is well established that excess body fat correlates with dysfunctional metabolism and elevates the risk of Type 2 diabetes (T2D), cardiovascular disease, Alzheimer´s disease and certain types of cancer [2–4]. Since the first studies highlighted a local inflammation in obese adipose tissue [5, 6], further research confirmed that a steatosis driven persistent state of low-grade inflammation, triggered by modern lifestyle factors such as overnutrition, low dietary fiber, sugar-rich diet, physical inactivity, vitamin D deficiency, psychosocial stress and sleep deprivation, is the common denominator of Western diseases (WD) [7–13].

Western diseases typically emerge when industrialization and wealth progresses and lifestyle becomes sedentary. The metabolic maldevelopment can be masterfully observed by the rate of obesity in South Pacific states such as Nauru (92.8%), Cook Islands (90.6%) and Tonga (88.1%), leading the list of obesity in the world [1]. Other indigenous cultures, recently transforming towards a Western lifestyle, reveal similar trends [14–18]. Noticeably, the rapidly increasing prevalence of T2D in indigenous people is much higher in urban than in rural areas [19, 20], due to the easy access to processed high glycemic load foods concurrent with fundamental alterations in the social and working environment, leading to chronic psycho-emotional stress and physical inactivity. The shift from a native to a modern sedentary lifestyle reflects the origin of metabolic diseases and displays the severe mismatch between the slow process of natural selection and the ultra-rapid changes in developing countries [21, 22].

Until the advent of the Agricultural revolution around 12,000–10,000 BC, humans had to cope with life-threatening fluctuations in energy supply. Adapting the sustenance on various climate and vegetation terms supported humans to explore new horizons to satisfy their basic needs of hunger, thirst, curiosity and preservation of the species. Daily survival of our Paleolithic ancestors was characterized by seasonal food availability, adaptation to a widespread range of different food sources, abundant daily fasting exercise, as well as an unpredictable provision of energy, depending on the foraging success. As a consequence, humans were constrained by environmental pressure to develop an extraordinary flexible metabolic system in order to guarantee a constant energy substrate influx. Gluconeogenesis, lipogenesis, ketone body utilization and the use of lactate as an alternative energy substrate are examples by which evolution tailored humans to buffer fluctuations in energy supply.

For approximately 84,000 generations humans lived under hunter and gatherer conditions [23, 24]. Since the agricultural (12,000 years ago ≈ 350 generations), the industrial (150 years ago ≈ 7 generations) and the digital revolution (only 30 years ago ≈ 2 generations), which in total represent only 0,5% of our existence, humans have dramatically changed their lifestyle, as compared to the current knowledge from our Paleolithic ancestors and the few remaining hunter-gatherer societies [25–28]. Today, Western people live indoors most of the time, consume their meals before they move, consume processed, high glycemic load-foods with artificial additives and preservatives, ingest energy permanently during daytime with a physical activity level failing to reach even 50% of recent foragers [24, 29, 30].

Our metabolic system was evolutionary designed for unpredictable environmental conditions rather than a constant availability of nutrients, which nowadays entails adverse metabolic consequences such as hyperglycemia, hyperinsulinemia and dyslipidemia [3, 31, 32]. Abnormal and consistent fat accumulation correlates with inflammatory diseases. It has now become clear that low-grade inflammation (LGI) is the breeding ground of most WD [3, 31, 33–35].

In contrast to monocausal interventions such as exercise programs to reduce body weight, bariatric surgery, calorie restricted diets or pharmaceutical agents, our study is based on the synergistic effects of fasting exercise (foraging behavior), unpredictability of food intake (foraging success and seasonal availability), low meal frequency (intermittent fasting) and absence of modern stressors, such as permanent accessibility by mobile phones, time pressure, physical inactivity, toxic compounds of processed food (e.g. gluten, casein), industrial chemicals (e.g. cosmetics, plastics) and environmental toxins – as far as possible. The goal of the present study was to evaluate which metabolic changes will occur if humans reset their modern behavior patterns towards a Paleolithic lifestyle, spending their daily routine hunting and gathering food instead of earning money, gaining social acknowledgement or other social-driven motivations.

Methods

Design

Our study protocol is in accordance with the declaration of Helsinki and has been approved by the local ethics committee. All participants provided written informed consent prior to their participation. Following the track towards an original way of life, we personally explored the hunter and gatherer lifestyle in a 10-day setting in the Spanish Pyrenees in 2011 as a self-experience trip. Regarding the unpublished findings at that time, we perceived that a 10-day intervention would probably not be necessary to normalize the metabolism of sedentary modern people. Accordingly, we initiated the current Eifel study, commenced by a small pilot group in 2013 [36], followed by this upgrade including a larger cohort. In this observational study, we have changed not only the typical Western diet regimen towards the Paleolithic prescription proposed by Cordain *et al.* [27, 37–42], but also other Western lifestyle factors based on the following modifications:

- No processed foods
- Unknown food supply (unknown type of food and consumption time)
- No soft drinks or beverages (only water provided)
- Food intake only twice a day, around noon and before sunset
- One fasting period >12 hours per day (8 hours of sleep /4 hours of hiking))
- Living 24 hours in the open air with a tribe of 14 people, sleeping in the open air
- Hunting before consuming any meal (hiking under fasting conditions for at least 3–4 hours per day)
- Cut off from the outside world
- Cut off from modern stressors such as time pressure
- Temperature control (only modest clothing for changing and a sleeping bag available)
- Natural biorhythm (natural day-night cycle according to the sun)
- Staying in the woods most of the time

Within these framework parameters, we attempted to imitate Paleolithic conditions as accurately as possible. Considering that the Asian forest bath studies [43–46] could impressively underline the positive impact on several health parameters, resulting from short hiking trips in woodlands compared to walks in a city, we laid our main focus on metabolic parameters such as the homeostasis model assessment-index (HOMA) and the novel fatty liver index (FLI), while taking into account any changes in the subjects' body composition and blood values including the acute phase inflammation marker high-sensitive C-reactive protein (CRP). Although not measured in daily routine, HOMA gains influence in clinical diagnostic, concerning the metabolic syndrome (MeS) and T2D, while the FLI is virtually unknown. Given the fact that there are healthy and unhealthy overweight people, body mass index, waist-to-hip ratio and other non-invasive standard measurements could be indicators for a metabolic risk, but fail to detect them reliable [47]. For that reason, we included HOMA in order to investigate the impact of our intervention on insulin resistance (IR). By means of the FLI, an easy-to-use instrument developed by the Italian group of Bedogni [48–50], we wanted to prove whether such a short comeback to our Paleolithic past has the potential to decrease ectopic liver fat. This is an exceptional metabolic aspect in the light of a growing population, evolving towards a non-alcoholic fatty liver disease (NAFLD). Meanwhile, 40–50% of Westernized people suffer from a NAFLD, which is highly correlated with MeS, cardiovascular disease and T2D [47, 51].

Subjects

Participants were recruited from advanced training courses of the German Trainer Academy in Cologne, composed of personal trainers, health professionals and healthy non-obese volunteers. All participants accepted Jens Freese and Sebastian Schwarz as the coordinators of this study. They completed a 60-minute on-site introductory seminar about the main principles of our study design. Considering nature, subjects were randomly divided in two groups and introduced to their guides, who already participated in the pilot study one year before. As a result, they were experienced in the procedure, tracks and region. Exclusion criteria of the study were chronic medication use, acute injuries and psychiatric disorders.

Two groups of 14 volunteers were isolated for a period of four days and four nights in the national park DELUX on the border between Germany and Luxembourg. Subjects lived outdoors without tents, although guides were equipped with tarps to avoid getting wet during the night. Groups hiked apart from each other, not knowing the direction, times of rest and times of food intake. Every participant carried a defined small day package of fruits and nuts with the instruction not to eat before noon (Figure 1).

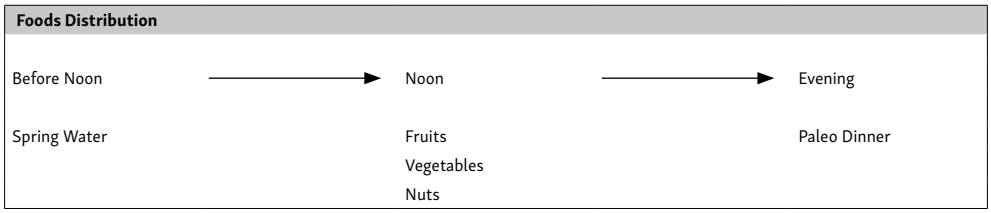

Figure 1. Foods distribution during the intervention

The intention of the present study was to guarantee an intermittent fasting period of at least 12 hours once a day, achieved by 8 hours of sleep and 4 hours of fasted hiking in the morning.

Seasonal, organic foods were locally bought in order to achieve maximum freshness. Coordinators prepared food for dinner in the nearby rural hotel, based on the Paleolithic diet recommendations from Cordain *et al.* [27, 38, 41] (for a list of our food choices see Table 3). Before cooking, all foods were accurately measured by weight (grams) for the later energy intake assessment. After the guides had chosen the groups' sleeping location in the woodlands, dinner was directly delivered to them by car. In order to avoid any environmental impact, dinner plates and waste were collected after each meal.

Data collection

Blood samples (lithium-heparin and potassium oxalate/sodium fluoride tubes) and EDTA-anticoagulated blood tests were drawn by venipuncture from fasted subjects the first morning and on day four immediately after the morning hike and during fasting conditions. Blood samples were drawn by a medical doctor and drained into three tubes: Sarstedt S-Monovette Serum-Gel (clinical chemistry), Sarstedt S-Monovette Kalium-EDTA (blood panel) and Sarstedt-S-Monovette Natrium-Fluorid (glucose). The tubes were stored in a cooling bag and immediately driven to the Dr. Quade & Kollegen laboratories in Cologne. Complete blood cell-count was analyzed with the XN 2000 Sysmex (Sysmex GmbH, Norderstedt, Germany). Quantitative and proportional analysis of the examined blood components were determined by electric impedance, laser light dispersion and dye binding. Gamma-glutamyltransferase, triglycerides, cholesterol, high density- and low-density lipoprotein were determined with the ADVIA 1800 Siemens (Siemens Healthcare GmbH, Erlangen, Germany) by the IFCC-method. Glucose was measured by the hexokinase reaction. Insulin was analyzed by chemical luminescence immunoassay reaction and CRP by latex-enhanced-immunturbidimetric assay with the ADVIA 1800 Siemens. Bioelectrical measurements were performed using the Tanita Weight Management System MC 780MA S (Tanita Europe B.V, Amsterdam, The Netherlands) and carried out immediately after blood tests. They were also drawn on the first and the last day of the intervention.

For the quantification of the daily hiking distance, we used the portable navigation system Etrex Vista HCX (Garmin International, Olathe, USA). Comprised with a high-sensitive GPS receiver, group's position was located in each environment. A built-in compass was used for navigation in the woods. To gain insight into the participants' daily physiological output and sleep behavior, SenseWear® armbands (BodyMedia Inc., Pittsburgh, USA) were applied. One male and one female subject wore an armband on the back of the upper left arm, facing upwards towards the shoulder with the sensors touching the skin. Due to the fact that groups spent every minute together, we only implemented two armbands exemplarily. The system records physiological parameters and uses algorithms to report the average daily activity and sleeping duration. Of the two samples, mean values were calculated and divided by the number of days in order to identify the approximate daily average for each measurement.

Statistical analysis

Statistical analysis of all measurements before (pre) and after (post) excursion were made using student t-tests for dependent samples using Microsoft Excel 2013 and SPSS (version 16.0).

Results

Demographic profile

A total of 12 females and 13 males completed the present study. Three participants dropped out after

Table 1: Demographic and anthropometric features of all participants.

Items	Participants
Race	Caucasian
N	25
Female	12 (48%)
Male	13 (52%)
Age (years)	40 (± 13)
Normal weight BMI < 25 (kg m^2)	20 (80%)
Overweight BMI > 25 (kg m^2)	5 (20%)
Exercised trained (> 3 h/week)	12 (48%)
Not exercised trained (< 3 h/week)	13 (52%)
Smoker	0

Table 2. Physiologic output and sleeping behavior during the intervention.

Items	Results
Sleeping Time per Day	7.15 h
Activity Time per Day	5.49 h
Distance per Day	16.4 km
Steps per Day	24,963

one night due to personal stress load concerning the study's program design. The mean age of subjects was 40 (± 13) years, with a range of 25 to 70 years. Twenty subjects (80%) were classified as normal weight and 5 (20%) as overweight. One subject was a smoker. Of 25 subjects, 12 were classified as exercise-trained (Table 1). Subjects were classified as exercise-trained if they regularly performed three hours per week of anaerobic and aerobic exercise with moderate to high intensity. Collectively, subjects were relatively healthy, and did not rely on any prescription drugs. Eligibility and classification were determined by completion of a pre-admission questionnaire.

Physical Activity Level

Physiological output and sleeping behavior, measured by SenseWear® armbands, revealed a daily average physical activity of 5.49 h, a sleep duration time of 7.15 h and 24,962 steps taken (Table 2). GPS data reckoned 64.84 km in 4 days, which means 16.21 km of hiking distance per day.

Caloric Profile

For the estimation of the total caloric intake and macronutrient ratios, the USDA Nutrient Interactive Database [52] was used. Daily energy intake for each participant reached an average of 1,855 kcal. The ratios of macronutrient intake during the intervention were calculated with 26% carbohydrates, 49% fat and 25% protein (Table 3).

All foods were measured by weight (grams). To estimate total caloric intake and macronutrient ratios, we used the USDA Nutrient Interactive Database [52].

Anthropometric and Biochemical Measurements

After 4 days of simulated Paleolithic conditions body weight (–2.9 kg), BMI (–2.7%), body fat (–10.4%), visceral fat (–13.6%) and waist/hip-ratio (–2.2%) decreased significantly (Table 4). Muscle mass increased significantly (+2.3%), although participants performed mainly low impact movement and the daily caloric intake (1,855 kcal/day) was above the basal metabolic rate of all subjects. To study the impact of a Paleolithic lifestyle change on human metabolism (Table 5), we measured selected parameters, relevant for the early detection of MeS. We found a significant decrease in fasting glucose (–6.5%) and basal insulin (–44.4%), which in total lead to a significant decrease of HOMA (–49.3%). Further, the FLI also decreased (–41%) significantly. In contrast to all measured metabolic parameters, CRP, which represents the first immunological response to inflammatory conditions, increased (+67.1%) excessively.

Discussion

The purpose of the present observational study was to investigate if a short comeback to Stone Age conditions has the potential to counteract the slinking metabolic diathesis of an expanding sedentary society. O´Dea *et al.* showed more than 30 years ago in diabetic urban Aboriginal Australians [53] that glucose metabolism normalizes and IR disappears after a 7-week reversion to a hunter-gatherer lifestyle in their natural habitat. Lindeberg demonstrated that the mean insulin concentration of primal living Kitava islanders between 50 to 74 years was 50% lower compared to Swedish subjects following a typically Western lifestyle. Further, mean insulin levels decreased with age in the Kitava group and increased with age in the Swedish group [25, 39, 40].

Although humans' metabolic system depends on a sufficient replenishment of macro- and micronutrients, it is extremely flexible to overcome multiple environmental circumstances e.g. famine, seasonal abundance of food, climate changes, long journeys without food availability, rapid escape from predators

Table 3. The choice of foods as listed was consumed over 4 days.

Foods	Weight (kg)	Kcal	Protein (g)	Fat (g)	Carbo-hydrate (g)
Almond	1.5	8,685	317.25	748.95	323.25
Apple	19.9	1,040	5.2	3.4	276.2
Apple Cider Vinegar	0.3	63	0	0	2.79
Apricot	4.9	2,352	68.6	19.11	544.88
Eggplant	3.45	1,225	29.05	8.05	305.55
Avocado	0.4	640	8	58.64	34.12
Banana	16.13	14,356	175.82	53.23	3,684.09
Broccoli	1.4	476	2.57	5.18	92.96
Canola Oil	0.65	5,693	0	644	0
Carrot	13.5	5,535	125.5	32.4	1,293.3
Celery	0.6	96	4.14	1.02	17.82
Cashew	0.4	2,212	72.88	175.4	120.76
Chicken thigh	10.12	23,664	2,372.52	1,500.42	0
Cabbage	5	1,250	64	5	290
Dark chocolate	0.8	4,632	48.96	306.48	419.36
Eggs	100	7,150	625.5	473.5	35.5
Ginger	0.1	80	1.82	0.75	17.77
Ground Beef	6	13,800	1707	722.4	0
Honey	0.22	1,216	1.2	0	329.6
Leek	2	620	16.2	4	152.4
Beef, lean, cooked	6.278	13,608	1,924.02	658.98	0
Mushrooms	3.6	792	111.24	12.24	117.36
Nut mix	4.87	24,255	1568	1862	539
Olive Oil	0.95	7,638	0	864	0
Onion	3.9	1,560	42.9	3.9	364.26
Brazil nut	0.8	5,248	114.56	531.44	98.16
Peach	3	1,170	27.3	7.5	286.2
Pepper sweet	2.3	713	22.77	6.9	138.69
Radishes	3.2	512	21.76	3.2	108.8
Salmon, wild, cooked	6	11,040	1,641.6	450	0
Spinach	1	230	28.6	3.9	36.3
Strawberry	1.9	608	12.73	5.7	145.92
Swiss Chard	0.35	70	6.58	0.28	14.46
Turnip	4.7	1,316	42.3	4.7	302.21
Walnut	1.27	8,034	312.78	767	128.83
Watermelon	24	7,200	146.4	36	1812
Zucchini, squash	4	760	40.4	10.8	155.2
Daily kcal/person		**1,855**			
Macronutrient ratios			**Protein 25%**	**Fat 49%**	**Carbohydrate 26%**

etc. [54, 55]. Hence, in the course of evolution, humans were constrained to develop various metabolic strategies for different survival situations, which predominantly cover the need of its metabolically most expensive organ, the human brain [56, 57]. Consequently, cerebral neurons, which are incompetent to store energy substrates, show an extraordinary flexibility in shifting their energy production from glucose as the primary source, to either ketone bodies or lactate, according to whatever is available to guarantee a constant energy provision. In this way, the human brain has become independent of continuous fueling with glucose. Today, due to the excessive energy-dense food availability in a nearby environment, modern humans have unlearnt the metabolic capability to allocate energy from the periphery to the central nervous system [58], recognizable in characteristic symptoms of neuroglycopenia [59], which

Table 4. Changes in anthropometric data over the course of the intervention.

Body Composition	Pre	Post	Change	p
Body Fat (%)	20.171 (±7.38)	18.067 (±7.99)	2.104 (-10.4 %)	<0.001
Visceral Fat (cm²)	4.583 (±2.9)	3.958 (±2.77)	0.625 (-13.6 %)	<0.001
Weight (kg)	74.55 (±13.5)	72.421 (±13.01)	2.129 (-2.9 %)	<0.001
BMI (kg/m²)	23.675 (±2.82)	23.025 (±2.72)	0.65 (-2.7 %)	<0.001
Fat Free Mass (kg)	59.429 (±11.77)	59.35 (±12.43)	0.079 (-0.1 %)	0.83
Muscle Mass (%)	75.642 (±6.78)	77.825 (±7.63)	2.183 (+2.3 %)	<0.001
Total Body Water (%)	56.729 (±5.29)	58.538 (±6.06)	1.808 (+3.2 %)	<0.001
Waist (cm)	83.667 (±9.32)	81.792 (±9.22)	1.875 (-2.2 %)	<0.001
Waist/Hip-Ratio	0.833 (±0.08)	0.814 (±0.08)	0.019 (-2.2 %)	<0.001

Values represent the mean ±SD. P-values shown are uncorrected.

Abbreviations: BMI (body mass index), ICW (intracellular water), ECW (extracellular water)

Table 5. Changes in biochemical data over the course of the intervention.

Biochemical Data	Pre	Post	Change	p
Leukocytes (nl)	6.105 (±1.54)	5.908 (±1.49)	0.197 (-3.2 %)	0.558
Erythrocytes (PL)	4.848 (±0.36)	4.669 (±0.41)	0.18 (-3.7 %)	<0.001
Hemoglobin (g/dl)	14.821 (±0.93)	14.204 (±1.02)	0.617 (-4.2%)	<0.001
Hematocrit (%)	42.633 (±2.47)	41 (±2.98)	1.633 (-3.8 %)	<0.001
MCV (fl)	88.096 (±3.5)	88.004 (±3.59)	0.092 (-0.1 %)	0.729
MCH (pg)	30.613 (±1.42)	30.496 (±1.45)	0.117 (-0.4 %)	0.328
MCHC (g/dl)	34.758 (±0.66)	34.654 (±0.72)	0.104 (-0.3 %)	0.289
RDW (%)	12.783 (±0.68)	12.533 (±0.7)	0.25 (-2 %)	<0.001
Thrombocytes (nl)	240.125 (±47.34)	242 (±48.39)	1.875 (+0.8 %)	0.672
Neutrophils (%)	54.833 (±8.81)	58.575 (±6.69)	3.742 (+6.8 %)	0.031
Lymphocytes (%)	33.267 (±8.14)	29.688 (±6.46)	3.579 (-10.8 %)	0.022
Monocytes (%)	8.538 (±1.94)	9.117 (±1.79)	0.579 (+6.8 %)	0.088
Eosinophils (%)	2.667 (±1.93)	1.933 (±1.18)	0.733 (-27.5 %)	0.014
Basophils (%)	0.696 (±0.24)	0.688 (±0.29)	0.008 (-1.2 %)	0.865
Fasting glucose (mg/dl)	80.342 (±8.84)	75.096 (±7.87)	5.246 (-6.5 %)	0.017
Gamma GT (U/L)	20.708 (±15.22)	20.125 (±12.64)	0.583 (-2.8 %)	0.365
Total Cholesterol (mg/dl)	200.5 (±32.93)	188.25 (±33.21)	12.25 (-6.1 %)	<0.001
HDL (mg/dl)	75.571 (±21.4)	78.296 (±19.83)	2.725 (+3.6 %)	0.056
Triglycerides (mg/dl)	95.625 (±78.71)	49.042 (±18.7)	46.583 (-48.7 %)	0.004
LDL (mg/dl)	113.113 (±28.28)	101.529 (±23.43)	11.583 (-10.2 %)	<0.001
LDL/HDL-Quotient	1.605 (±0.58)	1.363 (±0.4)	0.242 (-15.1 %)	0.002
CRP high sensitive (mg/l)	1.334 ±(2.623)	2.229 (±2.957)	0.895 (+67.1%)	0.121
Insulin (uU/ml)	8.943 (±7.52)	4.973 (±2.11)	3.969 (-44.4 %)	0.016
HOMA-Index	1.847 (±1.83)	0.937 (±0.46)	0.91 (-49.3 %)	0.022
Fatty Liver Index	20.51 (±23.9)	12.097 (±15.3)	8.413 (-41 %)	<0.001

Values represent the mean ± SD. P-values shown are uncorrected.

Abbreviations: MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red blood cell distribution), Gamma GT (gamma-glutamyl transpeptidase)

Paleolithic hunter and gatherer could presumably not afford in a life-threatening biosphere. In affluent societies, a decreasing blood glucose level is generally responded by food intake, ending up in enhanced meal frequency, caloric overnutrition, physical inactivity and subsequently cause severe metabolic disturbances such as MeS and TD2 in the long run [60, 61].

For those reasons, the present study's hypothesis that human's metabolic system, customized through more than 3 million years of multifaceted

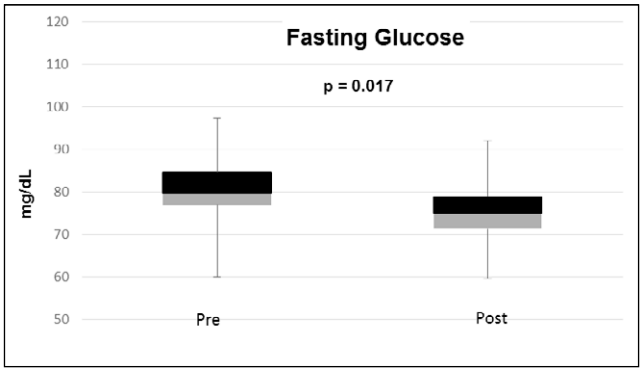

Figure 2. Boxplots showing fasting glucose at baseline (Pre) and post intervention (Post).

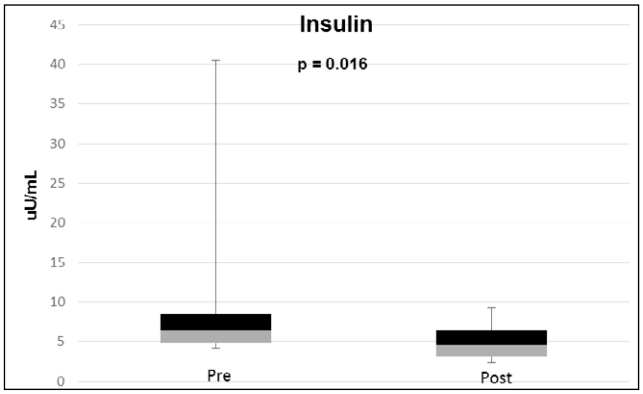

Figure 3. Boxplots showing insulin at baseline (Pre) and post intervention (Post).

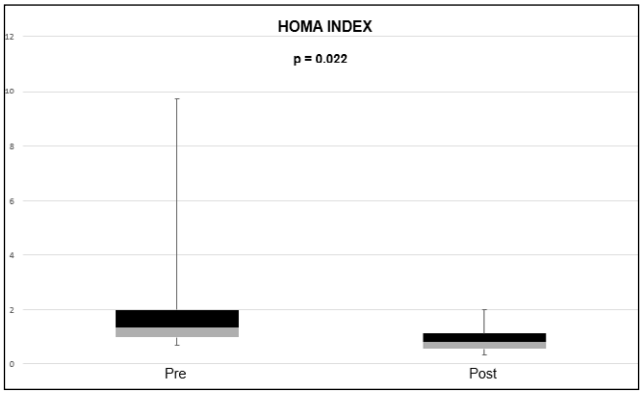

Figure 4. Boxplots showing HOMA-Index at baseline (Pre) and post intervention (Post).

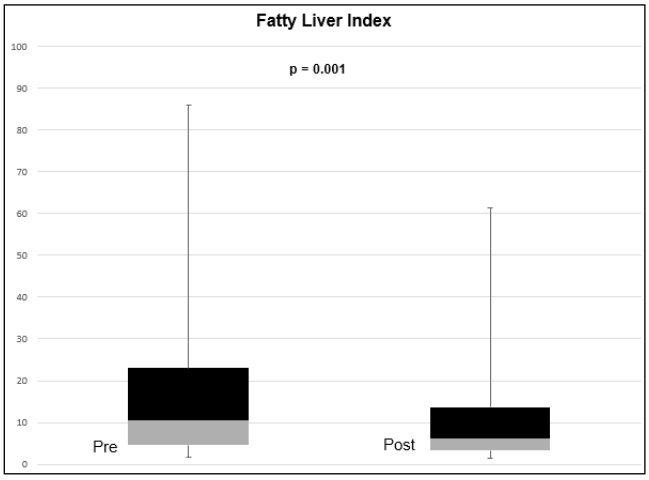

Figure 5. Boxplots showing Fatty Liver Index at baseline (Pre) and post intervention (Post).

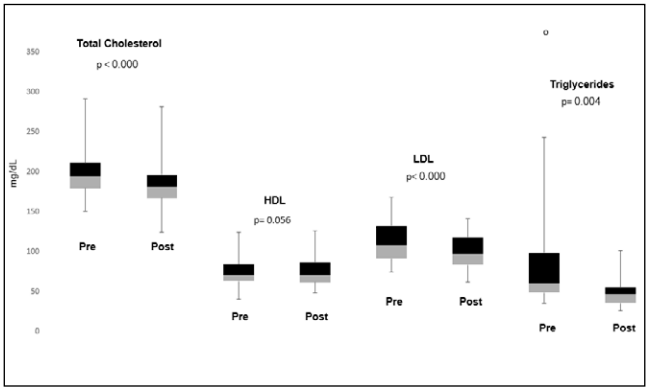

Figure 6. Boxplots showing total cholesterol, HDL, LDL and triglycerides at baseline (Pre) and post intervention (Post).

environmental circumstances, should be able to reactivate its primal flexibility within a short timescale, if all lifestyle parameters (Table 6) to a Paleolithic-like framework were set back. As a result of the study design, participants were compelled to imitate life as a hunter-gatherer in terms of intermittent fasting conditions (two meals per day), choice of food (Paleo diet), physical activity (most of the time under fasting conditions) and other natural pursuits, such as orientating in the wild or observing plants and animals (Figure 3).

In comparison to the pilot study of Freese *et al* [36] a mean of 288 kcal/day (1,855 kcal/day) was calculated above the initial trial (1,567 kcal/day). Interestingly, with this caloric surplus, the reduction of all measured metabolic parameters (Figures 2–6) was even more significant than in the pilot study one year before [36]. Therefore, the current results confirm that a calorie-reduced diet below the basal metabolism should not be recommended to decrease excessive body fat. On the contrary, time-restricted weight management programs under sedentary terms promote a severe state of IR, pre-diabetes, hepatic steatosis, and lipotoxicity in the longer term [49, 62, 63]. Taking into account that 12 out of 25 participants work as professional personal trainers, the dimension of the current findings, especially in the context of HOMA, FLI and blood lipids, is even more striking.

Although not measured, it is presumed that either keeping the energy supply above the basal metabolic rate or the stress reducing impact of the forest environment (or both) provided a lower output of stress hormones. The forest bath studies in Japan and China impressively displayed that walking in the woods promotes lower concentrations of cortisol, heart rate, blood pressure and sympathetic nerve

Table 6: Sedentary lifestyle to simulated Paleolithic conditions changes over the course of the 4-day intervention.

Conditions	Activities
Physical	Hiking
	Swimming in a River
	Climbing
	Collecting and Lifting Wood
External 1	Reduced Calories
	Less Frequent Food Intake
	Intense Sunlight
	Exposure to Mosquitoes and Ticks
	High Temperatures During the Day
	Cool Temperatures Morning and Nights
Internal	Feeling Thirsty
	Feeling Hungry
	Sweating and Freezing
	Aching Muscles
Other	Building a Fire
	Getting to Know the Group
	Searching for Suitable Night Camp
	Orientating in the Forest
	Looking for Wild Foods
	Watching Wildlife
	Sunbathing

activity [40, 44, 45]. Likely, in the absence of modern stressors, participants could have unfolded a greater fat oxidation rate with a boosted ketone metabolism (not measured). In association with a moderate supply of carbohydrates from fruit and the uncommon amount of hiking activity under fasting conditions in combination with other anthropogenic factors (e.g. no time pressure), possibly explain the ultra-rapid decrease in body fat (-10.4%), visceral fat (-13.6%), triglycerides (-48.7%), and most notably the FLI (-41%). Considering that 10–35% of Western people suffer from a NAFLD [63, 64], the remarkable reduction of the FLI in only four days encourage to use brief outdoor trips based on humans' primal behavior patterns, rather than diet or exercise programs alone, to oppose the spreading metabolic epidemic.

Intriguingly, the acute phase protein CRP (+67,1%) increased exorbitant. CRP is produced by the liver in response to elevated concentrations of IL-6 distributed by macrophages and adipocytes in order to orchestrate a pathogen-induced inflammation. There are several plausible explanations for this phenomenon. The radical change from a more or less sterile modern lifestyle situation into a wild habitat could have stimulated the innate immune system to challenge „old friends", a term stated by Rook *et al.* [65], which characterizes pathogens such as bacteria, parasites or fungi, human beings have to cope with since millions of years. It should also be considered that human species Paleolithic ancestors were confronted with numerous safety hazards, e.g. unknown plants, insects or other potentially toxic compounds in unclean water or rotten meat. In further studies, it should be examined if participants who are used to live outdoors, show lower inflammatory reactions. Another explanation could be that natural substances from trees and other plants such as phytoncides (wood essential oils) stimulate humans' innate immune system, facilitating the resolution of a chronic low-grade inflammation state [43, 66, 67]. Evidence suggests that walks in forested but not in urban areas reduce inflammatory cytokines [68]. In this case, the intervention presented in the current study could serve as a potent preventive medicine.

The present study implies several limitations. By virtue of financial limitations, the persistence of these significant metabolic effects could not be proven. Follow-up studies should examine data after seven days and one month, in combination with cortisol day profiles, heart-rate variability and ketosis measurements as well as quality of life questionnaires. Due to the fact that metabolic changes are not expected in four days, a control group at this stage of the research was deliberately waived. Further investigations should compare the presented synergistic four-day program with an unpaired Paleo diet group and a hiking group in an urban park or on an indoor treadmill. Nevertheless, the present study offers initial evidence that sedentary modern people could benefit from short comebacks into humans' Paleolithic past.

Conclusions

In conclusion, this study indicates that short trips to simulated Paleolithic conditions, where modern sedentary humans readapt their behavior patterns on their primal habitat, as it has been for approximately 99.5% of humans' existence, could operate as an effective preventive strategy to face the insidious danger of metabolic diseases. Particularly, the major findings of an expeditious reduction of HOMA and FLI in a short duration of only four days may disclose sustainable benefits of a short but radical lifestyle change rather than long-term single interventions such as dietary or fitness programs.

Abbreviations

WD: Western diseases; T2D: Type 2 diabetes; LGI: Low-grade inflammation; NAFL: Non-alcoholic fatty liver; MeS: Metabolic syndrome; FLI: Fatty liver index; CRP: High-sensitive C-reactive protein; HOMA: Homeostasis model assessment-index; IR: Insulin resistance.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors´ contributions

JF conceived and designed the experiment. JF, SS performed the experiment. JF, RH, BR analyzed the data and performed statistical analysis. JF wrote the manuscript. All authors read and approved the final manuscript.

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CHAPTER 1.4

Influence of a 10 days mimic of our ancient lifestyle on anthropometrics and parameters of metabolism and inflammation. The 'Study of Origin'

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Abstract

Background: Chronic low-grade inflammation and insulin resistance are intimately related entities that are common to most, if not all, chronic diseases of affluence. We hypothesized that a short-term intervention based on 'ancient stress factors' may improve anthropometrics and clinical chemical indices.

Objective: Study whether a 10-day-mimic of a hunter-gatherer lifestyle favorably affects anthropometrics and clinical chemical indices.

Methods: Fifty-three apparently healthy subjects and two patients with fibromyalgia (22–69 years, 28 women), in 5 groups, engaged in a 10-day trip through the Pyrenees. They walked 14 km/day on average, carrying an 8-kilo backpack. Raw food was provided, self-prepared and water was obtained from waterholes. They slept outside in sleeping bags and were exposed to temperatures ranging from 12–42 °C. Anthropometric data and fasting blood samples were collected at baseline and the study end.

Results: We observed median decreases ($p \leq 0.002$) of body weight (−3.5 kg), BMI (−1.1 kg/m^2), hip (−3 cm) and waist (−5 cm) circumferences, glucose (−0.6 mmol/L), HbA1c (−0.1%), insulin(−4.7 pmol/L), HOMA-IR (−1.2 mmol*mU/L^2), triglycerides (−0.14 mmol/L), total cholesterol (−0.7 mmol/L), LDL-cholesterol (−0.6 mmol/L), triglycerides/HDL-cholesterol (−0.55 mol/mol) and FT3 (−0.8 pmol/L). Changes in anthropometrics were unrelated to changes in clinical chemical indices, except for FT4 and FT3. ASAT (11 IU/L), ALAT (6 IU/L), ASAT/ALAT ratio (0.14) and CRP (0.56 mg/L) increased, and their changes were interrelated.

Conclusion: Coping with 'ancient mild stress factors', including physical exercise, thirst, hunger and climate, may influence immune status and improve anthropometrics and metabolic indices in healthy subjects and patients with fibromyalgia.

Introduction

Chronic non-communicable diseases (CNCD), including diabetes type 2, cardiovascular diseases, autoimmune diseases, chronic fatigue, depression and neurodegenerative diseases, are the major causes of morbidity, work absence and invalidity. They may be responsible for 35 million out of 52 million annual deaths worldwide [1]. CNCD has recently become the major topic for the World Health Assembly. In 2013, the Lancet issued a special on CNCD [2, 3]. Treating patients with CNDC is complex and of limited availability in many countries because of costs, while its proximate treatment has many side effects. Compliance is often low (e.g. lipid lowering drugs, anti-hypertensives) and many interventions have proven unsuccessful [4].

The vast majority of CNCD are caused by unhealthy lifestyle and other anthropogenic factors. It seems that none of us is immune for the damaging effects of modern lifestyle [5, 6]. Not surprisingly, many of these diseases are preventable by changes in behavior, including nutrition, physical activity and coping strategies [7–10]. The anthropogenic factors responsible for the CNCD pandemic include physical inactivity, unhealthy diet (e.g. high energy density refined food, too low vegetables, fruits and fish), chronic psycho-emotional stress, insufficient sleep (loss of biorhythm) and environmental toxins, including smoking [5, 11–18]. All of these may be considered 'danger signals' that activate central stress axes [sympathetic nervous system (SNS) and hypothalamus-pituitary-adrenal gland axis (HPA)] and the immune system [19, 20].

Inflammation is characterized by the five classical symptoms of rubor, dolor, calor, tumor and torpor. Inflammation requires metabolic adaptations [12]. Overt injury and infection bring about short-term allostatic responses aiming at the removal of the infectious agent, engaging in repair, and, ultimately, recovering homeostasis in a highly coordinated fashion [21]. While ancient infectious challenges induce optimal responses with self-resolving capacity, anthropogenic inflammatory stimuli deriving from modern society provide us with weak and long-lasting immunological responses that take us to a condition of chronic systemic low-grade inflammation with accompanying chronic hypometabolic adaptations [12, 22, 23]. It has become clear that many, if not all, CNCD are characterized by a state of low-grade inflammation (LGI, [24, 25]) that comes along with (e.g.) insulin resistance, hyperleptinemia, cortisol resistance, subclinical hypothyroidism and nerve-driven immunity. Jointly, they are responsible for the gradual development of multiple comorbidities together referred to as the typically Western diseases of affluence [11, 26–31].

The absence of ancient immune challenges in current Western societies inspired us to hypothesize that acute stress from ancient danger signals causes redistribution of the immune system towards its evolutionary preferred locations, and thereby favorably affects the state of chronic systemic low-grade inflammation, normalizes stress axes activities, recovers rhythmic function and restores insulin-insensitive pathways. Mild stress factors may activate resolution responses based on survival mechanisms that originate from millions of years of evolutionary pressure. In this study we investigated whether such 'ancient stressors', provided by a 10-day trip through the Pyrenees, improved anthropometrics and various clinical chemical parameters of low-grade inflammation, stress and metabolic control in 53 apparently healthy adults and two patients with fibromyalgia. The objective was to provide proof of principle that humans can influence their immune and metabolic systems by exposure to ancient mild acute stress factors. Our intervention mimicked, to some extent, the 'conditions of existence' of ancestral and current hunting/fishing-gathering populations.

Subjects and methods

Study group

The participants were students, scientists, physicians and other health professionals who were engaged in clinical Psycho-Neuro-Immunology (PNI) courses throughout Europe. They were interested to experience the impact of ancient lifestyle on their own health and well-being and therefore jointly decided to engage in this study. No consent from a medical ethical committee was deemed necessary, for which we refer to the constitutional law of self-determination, in which people have a basic right to decide what they want to do with their health (included in the patients self-determination act, United States 1991 and the Council of Europe 2009). The participants were part of their own team, united in a research consortium (see acknowledgments). They covered their own expenses and there were no grants. They appointed one of us (LP) as the study coordinator. All volunteers signed an informed consent and all were informed about the

trip details. The Catalan Government and the local government of Tremp (Spain) gave permission to execute our study without any restrictions.

We included apparently healthy adults and two patients with fibromyalgia. The fibromyalgia syndrome was diagnosed by medical specialists. Exclusion criteria were cardiovascular diseases, psychiatric diseases and chronic use of medication for serious illnesses.

Study design

Groups of 11 subjects, at most, participated in this 10-day trip through the Spanish Pyrenees during the summers of 2011 (n=10), 2012 (n=32) and 2013 (n=11). The participants lived outdoors and walked from one water-source to another. Food was provided by the organization and with help of forest-guards from official institutes of the Catalan county. Food intake was planned before the trip, based on the average daily food intake by the traditionally living Hadzabe people in Tanzania (Supplementary Tables 1 and 2). The use of mobile phones or other electronic devices was not allowed.

The detailed activities and condition during the 10-day study were as follows:

- First day, arrival at the hospital of Tremp (Catalonia, Spain) with an air-conditioned bus from Barcelona (2.5 h drive). Participants were once again informed about the trip. Anthropometric data and blood samples were collected in the fasting state.
- Daily walking trips from waterhole to waterhole, with an average walking distance of about 14 km/day, including altitude differences up to approximately 1,000 m. The participants carried their own backpacks with an average weight of 8 kg. The trip took place in the part of the pre-Pyrenees with a maximum altitude of 1,900 meters above sea level.
- Participants consumed two meals daily. The first was provided by the organization halfway and the second on arrival at the camping site. Animals, including ducks, chickens, turkeys, rabbits and fish, were delivered alive and killed by the participants. Fish were caught with nets in the Noguera river. All foods were prepared on the spot by the participants. Nutritional details are in Supplemental Table 2.
- The participants slept outside in sleeping bags on small inflatable mattresses. Outside temperatures varied from 22 to 42 °C during daylight, whereas night temperatures varied from 12 to 21 °C. One group experienced a day of snow in the middle of July, which prompted the organization to provide hotel accommodations for a single night.
- Bulk (intermittent) drinking behavior was recommended by drinking as much as possible (up to satiety) after reaching a waterhole. The waterholes contained non-chloritized drinking water.
- Some manual work was done to clean mountain trails as agreed upon with the Catalan Government.

Anthropometric data, sample collection and analyses

Sixteen anthropometric and clinical chemical parameters were measured before departure to the Pyrenees and after return. Anthropometric measurements were measured by one of us (LP) at the hospital of Tremp. The subgroup of two participants with fibromyalgia syndrome was followed for more than two years. Fasting heparin-, oxalate- and EDTA- anti-coagulated blood samples were collected by venipuncture in the first morning and the morning of the 10th day at the study end. All samples were transported at 5 °C and processed within 1 h. Analyses were done in the clinical laboratory of the hospital of Tremp.

HbA1$_c$, total-cholesterol, HDL-cholesterol, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and glucose were determined with a Cobas c-501 analyzer (Roche, Madrid, Spain). Serum insulin was measured by chemo-luminescent assays, using Dxi-600 (Beckman, Barcelona, Spain) and Liaison XL (Diasorin, Madrid, Spain), respectively. High sensitivity C-reactive protein (CRP) was measured with a Behring Nephelometer II Analyzer System. HOMA-IR (mmol*mU/L^2) was calculated by glucose (in mmol/L)*insulin/22.5 (in mU/L) (Hill 2013)

Statistics

Statistical analyses were performed with IBM SPSS statistics version 23.0 (IBM Corp). Changes during the intervention were analyzed with the Wilcoxon signed rank test at $p<0.05$. Interrelations between variables were analyzed by Spearman´s correlation coefficient at $p<0.05$.

Results

Study group

There were 55 participants. Their median age was 38 years (range 22–67). The number of women was 28 (50.9%). Baseline anthropometrics are in Table 1.

Course of the trip

The 10-day trip went through the low and middle-high part of the Pyrenees. The majority of participants did very well. If one of them got too tired, LP or JA carried his/her backpack for as long needed. Trips were made from one waterhole to another, with a consistent midday pause of 1–1.5 h. The Mountainside of the Pyrenees is composed of hard soil and many of its vegetation carries thorns. Most participants suffered from small wounds on arms and legs, caused by the thorns of the aliaga, a plant belonging to the original vegetation of the Pyrenees. None of them suffered from infected wounds and most appeared fully cured at the end of the trip. Participants suffered from hunger feelings during the first three days, but gradually got used to eating only twice daily. Only one participant discontinued the trip, because she had different expectations. The local organization arranged transport and she left. The other participants enjoyed the trip for the full 10 days, without exceptions. Interestingly, the majority, including LP, wanted to go home after 7 days (see discussion).

Feelings of thirst were moderate at the beginning, but ameliorated after 3 days. Participants noticed that they could drink increasingly more water upon arrival at a waterhole and gradually experienced less needs for 'in-between water stops'. Three participants suffered from what might have been neuroglycopenic periods, feeling weak, hungry, cold, dizzy and trembling. However, measurement of their blood glucose level by finger prick did not reveal hypoglycemia. One of these participants was grossly overweight and exhibited fasting glucoses highly surpassing the normal range. He was not able to carry his backpack during the first three days, but managed surprisingly well during the last. At night, some participants were affected by the cold. They needed a thicker sleeping bag, which was supplied. Participants went asleep at sunset and rose at sunrise. Overall, participants felt good, occasionally tired, but not at all overloaded. Mosquitoes were a nuisance at night and therefore a liquid mosquito repellent was provided by the organization. Several participants suffered from diarrhea at the trip's end, probably from drinking water from the non-chloritized waterholes. Taken together, the vast majority of the participants enjoyed the trip and recognized the benefits by feeling more healthy and recovered from Western stressful life.

Anthropometrics and clinical chemical indices

Anthropometric and clinical chemical data collected before and after the excursion were available from 23–53 participants (Table 1). The missing values were attributable to the 2012 groups. Probably because of procedural imperfections in the Tremp lab, the insulin assay could not be performed in various series.

We found (Table 1) that body weight decreased with a median (range) of -3.8 kg (-12.5 to -0.7), BMI with -1.2 kg/m^2 (-4.4 to -0.2), hip circumference with -3 cm (-17 to +5), waist circumference with -5 cm (-18 to +9) and waist/hip ratio with -0.02 (-0.14 to +0.10).

We also observed decreases (median; range; Table 1) of: glucose (-0.6; -1.7 to +0.5 mmol/L), HbA1c (-0.1; -0,4 to +0.2 %), insulin (-4.7; -31.4 to -0.2 pmol/L), HOMA-IR (-1.2; -7.0 to -0.4 mmol*mU/L^2), triglycerides (-0.14; -6.12 to +2.18 mmol/L), total cholesterol (-0.7; -2.8 to +0.4 mmol/L), LDL-cholesterol (-0.6; -3.1 to +0.6 mmol/L), triglycerides/HDL-cholesterol ratio (-0.55; -8.98 to 1.34 mol/mol), and FT3 (-0.8; -3.4 to +3.1 pmol/L).

On the other hand we found that ASAT and ALAT activities increased with 11 IU/L (-8 to 54) and 6 IU/L (-13 to 52), respectively, while CRP increased with 0.56 mg/L (-15.72 to +41.07). Figure 1 shows the median and individual changes of ASAT (panel A), ALAT (panel B) and CRP (panel C). The ASAT/ALAT ratio before the intervention was 1.23 (0.68–2.00) and increased with 0.08 to 1.31 (0.48–2.06).

Figure 2 shows the medians of the percentage change for the anthropometric and clinical chemical parameters that were found to change significantly during the 10 days trip through the Pyrenees.

Two subjects with fibromyalgia

The two female participants (ages 37 and 38 years) were part of the 2011 group. Both managed surprisingly well. The first three days they still suffered from overall pain, but these gradually disappeared after 7 days. From the start, they preferred to carry their own backpacks but were in too much pain. LP and one of the others carried their backpacks most of time during the first three days. They were able to carry their own backpacks from then. Fatigue symptoms were severe during the first 4 days but diminished substantially after the fifth. Sleeping problems vanished almost immediately. Bowel movement was compromised at start, but improved during the trip

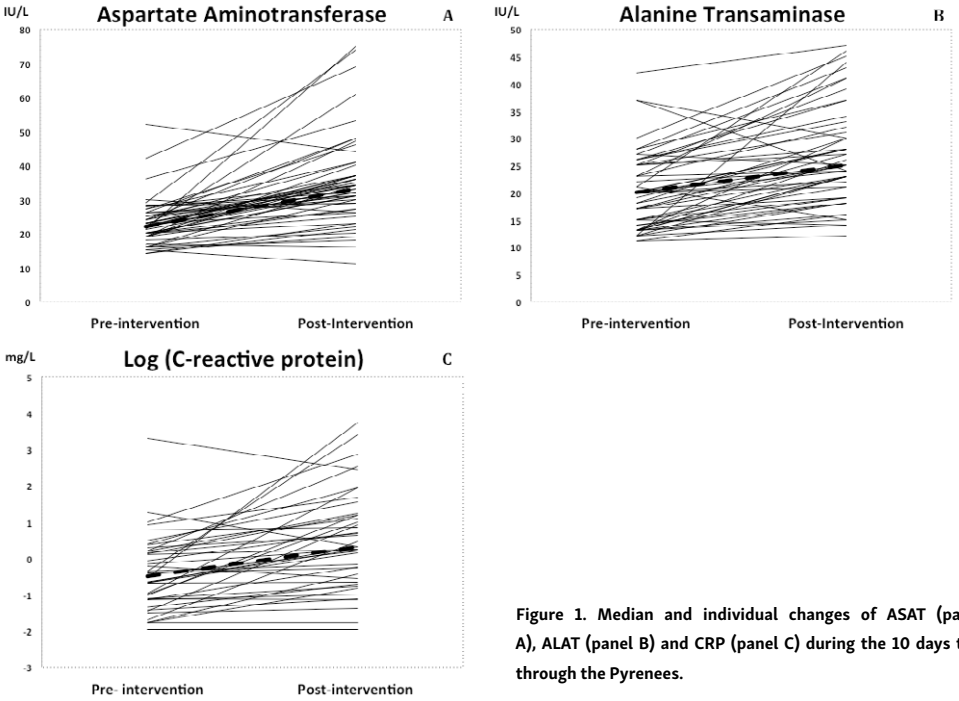

Figure 1. Median and individual changes of ASAT (panel A), ALAT (panel B) and CRP (panel C) during the 10 days trip through the Pyrenees.

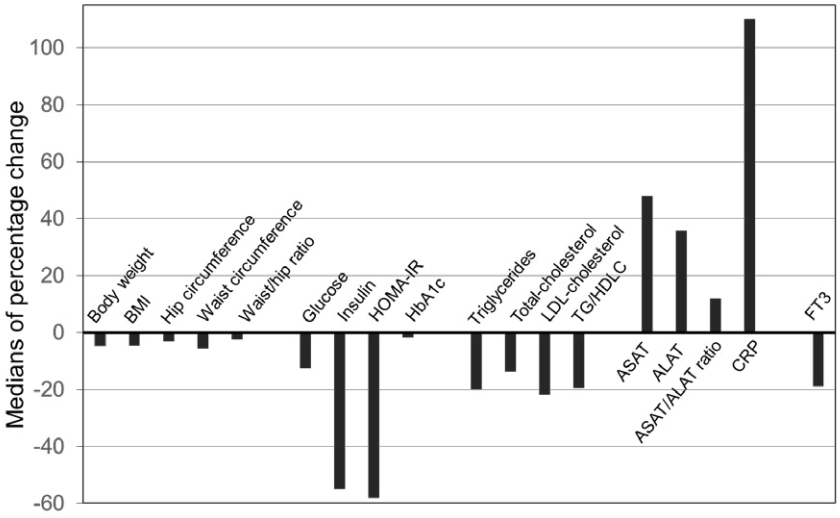

Figure 2. Medians of the percentage changes of anthropometric and clinical chemical parameters during the 10 days trip through the Pyrenees. Only significant changes are shown (see Table 1). For abbreviations see legend of Table 1.

to become almost normal in one of them at the study end. The second patient kept complaining about abdominal pain, but stool consistency and frequency were normal. No notable differences were observed in the changes of their clinical chemical indices when compared with other participants. Both showed

Table 1. Anthropometrics and clinical chemical indices at baseline and at the study end

	Unit	N	Baseline		Study end		Change				P
			Median	Range	Median	Range	Median	Range	SD	95% CI of the mean	
Body weight	kg	55	68.0	48.4–116.3	65.00	46.9–111.8	-3.8	-12.5 to -0.7	2.0	-4.4 to -3.3	<0.001*
Age	years	50	38	22–67	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
Height	cm	55	175	154–203	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
BMI	kg/m2	55	22.40	17.4–31.9	21.3	16.8–30.4	-1.2	-4.4 to -0.2	0.6	-1.4 to -1.1	<0.001*
Hip circumference	cm	44	100	85–120	96	86–115	-3	-17 to 5	3.3	-4.2 to -2.2	<0.001*
Waist circumference	cm	44	81	66–110	76	63–101	-5	-18 to 9	5.5	-7 to -4	<0.001*
Waist/hip ratio	cm/cm	44	0.84	0.72–1.00	0.80	0.66–0.94	-0.02	-0.14 to 0.10	0.06	-0.04 to -0.02	0.002*
Glucose	mmol/L	53	4.9	4.2–5.8	4.3	3.3–6.1	-0.6	-1.7 to 0.5	0.6	-0.8 to -0.5	<0.001*
HbA1c	%	53	5.3	4.8–6.1	5.3	4.7–6.1	-0.1	-0.4 to 0.2	0.2	-0.1 to -0.05	<0.001*
Insulin	mU/L	23	14.0	3.7–36.8	6.7	1.1–12.9	-4.7	-31.4 to -0.2	8.1	-12.2 to -5.2	<0.001*
HOMA–IR	mmol*mU/L2	22	3.0	0.8–7.9	1.4	0.2–2.6	-1.2	-7.0 to -0.4	1.8	-2.8 to -1.3	<0.001*
Triglycerides	mmol/L	53	0.69	0.34–6.68	0.52	0.37–2.77	-0.14	-6.12 to 2.18	0.92	-0.52 to -0.01	<0.001*
Total cholesterol	mmol/L	53	5.2	3.2–8.2	4.5	2.6–8.1	-0.7	-2.8 to 0.4	0.7	-1.0 to -0.6	<0.001*
HDL–cholesterol	mmol/L	53	2.0	0.7–3.1	1.9	1.0–3.5	0.0	-0.8 to 0 .6	0.3	-0.1 to 0.1	0.464
LDL–cholesterol	mmol/L	52	3.0	1.3–5.8	2.5	0.0–5.4	-0.6	-3.1 to 0.6	0.7	-0.8 to -0.5	<0.001*
TG/HDL–cholesterol ratio	mol/mol	53	0.3	0.16–9.54	0.26	0.11–1.73	-0.55	-8.98 to 1.34	1.3	-0.59 to 0.98	<0.001*
ASAT	IU/L	53	22	14–52	33	11–75	11	-8 to 54	11.4	9 to 15	<0.001*
ALAT	IU/L	53	20	11–42	25	12–47	6.0	-13 to 52	7.3	5 to 9	<0.001*
ASAT/ALAT ratio	IU/IU	53	1.23	0.68–2.00	1.31	0.48–2.06	0.14	-0.77 to 0.69	0.23	0.05 to 0.18	<0.001*
CRP	mg/L	42	0.61	0.14–27.04	1.36	0.14–41.65	0.56	-15.72 to 41.07	8.45	0.20 to 5.46	<0.001*
TSH	mU/L	42	1.25	0.02–3.12	1.11	0.02–4.40	-0.08	-0.93 to 1.28	0.47	-0.19 to -0.10	0.326
FT4	pmol/L	42	10.8	7.9–19.4	11.3	7.8–20.6	0.1	-5.6 to 8.4	2.3	-0.4 to 1.1	0.378
FT3	pmol/L	42	4.4	2.3–6.5	3.5	1.7–8.7	-0.8	-3.4 to 3.1	1.0	-1.0 to -0.5	<0.001*

Data are medians (range). Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; FT3, free triiodothyronine; FT4, free thyroxine; HbA1c, hemoglobin A1c; HDL, high-density-lipoprotein; HOMA-IR, homeostasis model assessment-estimated insulin resistance; LDL, low-density lipoprotein; N.M. Not measured; TG, triglycerides; TSH, thyroid-stimulating hormone. *, Significant difference between the values before and after the intervention by Wilcoxon signed rank test at p<0.05.

increases of ASAT (from 26 to 48; and 36 to 53 IU/L), ALAT (18 to 30; 25 to 39 IU/L) and CRP (0.37 to 2.71; 0.17 to 0.64 mg/L). while their HOMA-IR decreased (0.48 to 0.31; 1.04 to 0.52 mmol*mU/L^2). Their ASAT, ALAT and CRP values decreased during the months after the trip. Three months after the trip her ASAT had decreased from 48 to 22 IU/L in one of them, while ALAT decreased from 30 to 19 IU/L, and CRP from 2.71 to 0.5 mg/L. The other showed changes from 53 to 18 IU/L for ASAT, 39 to 16 IU/L for ALAT and 0.64 to 0.2 mg/L for CRP. Follow-up of both patients occurred during three years. At present both women are healthy. One became pregnant and mother of a healthy child. She started her own clinical PNI institute, while the other is completing an University master.

One subject with arrhythmia.

One of the participants suffered from cardiac arrhythmia since 2000, following a sternum fracture in a car accident. He was on medication since then. During the trip he stopped taking treatment and did not suffer from arrhythmic periods during the 36-month-period that passed since the study end.

Interrelationships

Relationships between changes of the anthropometric and clinical chemical indices are presented in Supplemental Table 3. Importantly, we found that the changes in weight, BMI, hip circumference, waist circumference and waist/hip ratio were generally unrelated to the changes of clinical chemical indices. Exceptions were the negative associations ($p<0.05$) between waist circumference and FT3 ($r=-0.359$), and waist/hip ratio and FT4 ($r=-0.360$). The change in HOMA-IR was negatively related to changes in total cholesterol ($r=-0.457$) and LDL-cholesterol ($r=-0.436$). Finally, we found that the changes in ASAT, ALAT and CRP were positively interrelated (ASAT vs. ALAT $r=0.777$; ASAT vs. CRP $r=0.508$; and ALAT vs. CRP $r=0.440$). Age proved positively related to the change of ASAT ($r=0.371$), but was unrelated to the changes of ALAT and CRP.

Discussion

The aim of this study was to investigate whether a 10-day trip through the Pyrenees favorably affects anthropometric-, metabolic- and inflammatory-parameters in apparently healthy subjects and two patients with the fibromyalgia syndrome. The trip mimicked to some extent the 'conditions of existence' of ancient and contemporary hunting-gathering populations. We found that the intervention was well tolerated and that all participants, except for one dropout, experienced a better subjective feeling of health following its completion. Also the two patients with fibromyalgia experienced improvements: they were able to meet the needed physical activity after 7 days. The pain vanished and favorable effects seemed to remain during a subsequent follow up of three years. The trip caused decreases in body weight and BMI (median change 4.8%), hip circumference (3%), waist circumference (5.6%) and waist/hip ratio (2.5%) (Table 1; Figure 2). Among the clinical chemical indices we found decreases of glucose (12.5%), insulin (55%), HOMA-IR (58.1%), HbA1c (1.8%), triglycerides (20%), total-cholesterol (13.7%), LDL-cholesterol (21.9%) and triglycerides/HDL-cholesterol ratio (19.3%). On the other hand, the medians of ASAT and ALAT increased with 48.0% and 35.7%, respectively, while CRP increased with 110.1%.

Favorable effects

Altogether we found that 3 features of the metabolic syndrome improved, i.e. body mass, glucose homeostasis and circulating lipids. The fourth, i.e. blood pressure, was not recorded. The metabolic syndrome, also named the insulin resistance syndrome, is a well-established risk factor for various diseases of affluence, including type 2 diabetes, cardiovascular disease, essential hypertension, polycystic ovary syndrome, non-alcoholic fatty liver disease, certain types of cancer (colon, breast, pancreas), sleep apnea and pregnancy complications, such as preeclampsia and gestational diabetes [32]. Although we did not aim at hard endpoints, our results suggest that the 10-day intervention could be of value to both primary and secondary prevention of the 'typically Western diseases of affluence'.

Potential mechanisms

Our intervention is based on causing 'mild acute stress' in humans who in their usual daily lives are exposed to the chronic stress commensurate with our modern lifestyle. Acute stress promotes release of stress hormones, including adrenaline, noradrenaline and cortisol, that each cause profound metabolic and immunologic adaptations [33]. For instance, a recent study by the group of Pickkers [34], showed that extreme cold exposure, combined with breathing exercise (producing intermittent hypoxia), profoundly

increases adrenaline secretion. This study and others [33, 35] show that acute stress factors increase autonomic activity, accelerate immune cell proliferation and differentiation, and also stimulate the anti-inflammatory component of the immune system (i.e. production of IL10, lactoferrin, lysozymes) [26]. Nevertheless, mild stress initially produces a pro-inflammatory response, which may subsequently give rise to recovery from the reigning state of chronic low grade inflammation and the return to homeostasis [36, 37].

In line with the above, we found that the observed changes in HOMA-IR and lipids were independent of weight loss, suggesting that a combination of lifestyle factzors might be at stake. Major, highly interacting, lifestyle factors contributing to typically Western diseases are poor diet, insufficient physical activity, chronic stress, insufficient sleep, abnormal microbial flora and environmental pollution (smoking included) [11, 38]. However, other mismatches with our ancient lifestyle are less appreciated. For instance, the participants suffered from thirst. Thirst relates to oxytocin production and the inhibition of stress axis activity [39, 40]. The participants were also disconnected from daily trouble and 'self-made' stress, which reduced the number of anthropogenic stress factors and possibly other inflammatory 'danger signals' [16]. Another factor might be the prohibited use of mobile telephones and other electronic devices. Although controversial, chronic mobile telephone usage may activate stress systems as evidenced by a recent study of Hamzany et al. [41]. Chronic use of mobile telephones also negatively affects the production of anti-inflammatory substances in saliva [42]. Absence of artificial light might be another factor. The trip forced the participants to adopt a 'natural day-night rhythm' in which the sleep-wake cycle was not dominated by social life, but rather by sunlight [43–45].

Spontaneous physical activity prior to food and water intake might be another beneficial factor. The postprandial inflammatory response has been identified as an independent risk factor for cardiovascular, metabolic and other non-communicable disorders [26, 46–48]. Pre-prandial exercise did not only blunt the pro-inflammatory response after food intake, but also produced a shift towards the production of anti-inflammatory mediators by the immune system and adipose tissue, conferring protection against possible pathogens present in food [49, 50]. Another important difference between Western life and the 10-day trip in the Pyrenees might be the presence of 'cutaneous- and other body surface- directed danger signals'. All participants suffered from little wounds on hands, arm and legs because of small injuries inflicted by sharp thorns and other natural obstacles. In addition, several participants suffered from mild gastrointestinal disorders and diarrhea. Fiuza-Luces et al. [51] attributed positive health effects to so-called hormetic triggers, including small external wounds and light muscle damage. Although speculative, the immune system might have migrated to sites that have been most susceptible to the damaging effects of the environment during evolution, including those affecting the skin, the gastrointestinal tract and the lungs, jointly being the sites with the highest need of immune surveillance [33].

Taken together, we propose that we are dealing with complex interacting lifestyle factors [5, 11] and that the current tendency to perform interventions aiming at single components, whether or not designed as RCTs, might suffer from a reductionist approach.

Possible adverse effects

We found increases of ASAT, ALAT and CRP, which at first glance might be regarded as genuine adverse effects. Increases of ASAT and the ASAT/ALAT ratio are related to cardiac muscle damage [52], while those of ALAT [53] and CRP [54] are intimately related to liver damage and infection, respectively.

Extreme sports, such as marathon and triathlon, are well known to elicit increases of lactate dehydrogenase, creatine kinase, ALAT, ASAT, ASAT/ALAT ratio, CRP and cardiac troponins [55–58]. The responses may easily exceed the upper limit of the reference range into the myocardial infarction area. The increases of cardiac troponins were similar when exercise was performed under controlled normoxic or hypoxic conditions, but proved dependent on exercise duration and intensity, possibly aggravated by hypoxic conditions [56]. Noakes et al. [59] also observed that novice runners had much higher responses than experienced runners and these results where confirmed in a later study [60]. We found that the augmentation of ASAT increased with age, as previously reported by Jastrzebski et al. [57]. Increases of cardiac markers under these conditions have not been firmly associated with irreversible cardiac muscle damage and are at present considered benign, at least in well-trained subjects. On the other hand, there are currently no data from long-term follow-ups [61].

The increases of both ALAT and CRP might also point at moderate liver damage and inflammation due to environmental exposure, the latter causing mild gastrointestinal infections from drinking water from waterholes, and small injuries on legs and arms inflicted by thorns and falls. Although the aforementioned plausible adverse effects will definitely require more attention in future interventions, they are not unexpected in the light of hunter-gatherer populations. For instance, the Pygmies exhibit huge gamma-globulin bands in the classical electrophoretic profiling of serum proteins, pointing at exposure to a host of different microorganisms and parasites [62]. Therefore, apart from the effects of intensive physical activity, the current findings remind us of the super-hygienic conditions of our current lifestyle. Hygiene is a major factor in longevity, but may, as a trade-off, also be at the basis of e.g. autoimmune disease; the so-called ´hygiene hypothesis´ [63–65]. For instance, a recent study in pregnant and newborn mice revealed that helminth colonization exerts beneficial effects on the infectious response of the offspring brain and also on microglial sensitization and cognitive dysfunction at adult life [66].

Comparison with previous studies

Our study is not the first to suggest that mimicking the lifestyles of traditional hunter-gatherer populations may be beneficial to our health. Already in 1984, O'Dea et al. showed that overweight Australian Aborigines with type 2 diabetes who reverted to their original lifestyle for 7 weeks, were able to improve, or even normalize, the characteristic abnormalities of diabetes, including improvements of body weight and fasting glucose, insulin and triglycerides. The favorable changes were attributed to weight loss, low-fat diet and increased physical activity [67]. Another example of the protective effects of our 'ancient conditions of existence' against modern lifestyle and 'Western diseases of affluence', may be compiled from the studies of the Kitava people in Papua New Guinea [68, 69]. Lindeberg et al. showed that these people, living a traditional lifestyle (e.g. consuming wild foods with profound physical activity) showed low rates of cardiovascular disease, obesity and other modern diseases, probably because of higher insulin sensitivity and lower levels of insulin, uric acid and leptin [70].

Limitations

Our study has many limitations. There was no control group and we also did not employ a cross-over or RCT-like design. The investigated group was relatively small and we only measured a small number of 'soft' parameters. Hard endpoints can obviously not be expected in short-term interventions in small groups with good general health. The findings of the two patients with fibromyalgia and the single subject with arrhythmia should be regarded as 'anecdotal'. However, whether a placebo effect or not, the subjective feeling of better health is certainly important and so are the observed changes in anthropometric and clinical chemical parameters. Our primary objective was to provide a proof of principle. More studies with more participants are certainly needed, including the recording of more parameters to objectivize e.g. the feeling of improved health. Also the minimum duration and intensity needed to demonstrate favorable effects are uncertain as yet.

Conclusions

The outcome of this 10-day 'Study of Origin' suggests that a short period of return to the 'conditions of existence' similar to those on which our genome is based may improve anthropometrics and metabolism by favorably challenging the immune system in apparently healthy subjects and possibly patients with fibromyalgia. The 'return' may come with some costs in more active infection, as a trade-off for the 'chronic systemic low grade inflammation' typical of our current lifestyle of affluence. We may increasingly appreciate that we cannot have it all, while the evolutionary lessons of Darwin and intervention studies [71] teach us that prevention might be more rewarding and affordable than the current culture of medical treatment.

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Supplementary Material

Supplemental Table 1. Average food intake of Hadzabe hunter-gatherers

Food consumed	Hadza en%	kcal
Meat	25	625
Tubers	17	425
Wild Berries	25	625
Fruits		
Eggs		
Fish		
Nuts		
Green Vegetables		
Baobab	11	275
Honey	22	550
Total	100	2,500

Data derive from Pontzer 2012, Murray 2001, IDF 2012, www.philosophy.dept.shef.ac.uk/culture&mind/people/crittendena/

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Supplemental Table 2. Average food intake of participants during the ten days trip through the Pyrenees (amount/10 persons)

Nutrient	Average amount	kcal / unit	total kcal	kcal/part /10 days
Banana	80	105	8,400	840
Apple	70	20	1,400	140
Melon	10 kilo	300	3,000	300
Mangos	4.5 kilo	645	2,900	290
Pineapple	3 kilo	460	1,380	138
Watermelon	8 kilo	280	2,260	226
Dates	1.1 kilo	2,760	3,036	304
Onions	12 kilo	400	4,800	480
Lettuce (6 types)	3 kilo	160	480	48
Cucumber	3 kilo	112	336	33
Garlic	2 kilo	132	264	26
Olives	1.5 kilo	1,660	2,500	250
Carrots	2.4 kilo	410	985	99
Mushrooms	1.1 kilo	225	250	25
Zucchini	1.5 kilo	160	240	24
Asparagus	1 kilo	220	220	22
Avocado	6 kilo	1,650	10,100	1,010
Pumpkin	2 kilo	200	400	40
Leek	2 kilo	550	1,100	110
Sweet potato	1.5 kilo	720	1,080	108
Coconut	5 kilo	3,180	15,900	159
Raisins	1 kilo	2,860	2,860	286
Berries	3 kilo	480	1,440	144
			Subtotal	6,532
Mayonaise	1 kilo	6,300	6,300	630
Olive oil	3 liters	8,400	25,200	2,520
Honey	2 kilo	7,000	14,000	140
			Subtotal	3,290
Deer	11 kilo	1,480	26,280	2,628
Chicken	9 kilo	2,140	19,260	1,920
Rabbit	2 kilo	2,764	5,528	553
Sweat water fish	8 kilo	1,590	12,720	1,272
Tuna in olive oil	5 kilo	4,030	20,150	2,015
Duck	6 kilo	2,016	12,096	1,209
Eggs	5.5 kilo	1,400	7,700	770
Nuts	3.7 kilo	575	20,275	2,027
			Subtotal	12,394
			Total/pp/trip	22,216
			Total/pp/day	2,222

Supplemental Table 3. Spearman's correlation coefficients for the relations between the changes in anthropometric and clinical chemical indices during the study

Body weight	Body weight	Height	Hip circum-ferene	Waist circumfer-ence	Glucose	Hb1A	Tri-glycerides	Total choles-terol	HDL-cho-lesterol	LDL-cho-lesterol	ASAT	ALAT	CRP	TSH	T4	T3	Insulin	BMI	HOMA-IR
Height	0.760**																		
Hip circumference	0.719**	0.414**																	
Waist circumference	0.712**	0.482**	0.674**																
Glucose	0.332*	0.174	0.248	0.232															
Hb1A	0.192	0.114	-0.052	0.175	0.479**														
Triglycerides	0.306*	0.111	0.039	0.389**	0.243	0.149													
Total cholesterol	-0.089	0.186	-0.258	-0.126	0.244	0.189	0.091												
HDL-cholesterol	-0.674**	-0.370**	-0.246	-0.504**	-0.096	-0.058	-0.312*	0.339*											
LDL-cholesterol	0.260	0.413**	-0.154	0.192	0.237	0.197	0.286*	0.809**	-0.224										
ASAT	0.496**	0.402**	0.267	0.544**	0.425**	0.390**	0.244	0.268	-0.370**	0.460**									
ALAT	0.519**	0.351*	0.425**	0.689**	0.553**	0.387**	0.430**	0.244	-0.317*	0.444**	0.842**								
CRP	0.355*	0.023	0.317	0.530**	0.083	0.251	0.470**	0.01	-0.420**	0.199	0.308*	0.305*							
TSH	0.134	0.199	0.011	-0.008	-0.106	-0.05	-0.056	-0.01	-0.205	0.144	0.182	0.082	0.282						
T4	0.063	-0.079	0.129	0.306	0.237	0.097	0.390*	0.121	-0.051	0.05	0.262	0.239	0.327*	-0.262					
T3	0.073	-0.112	0.606**	0.615**	-0.227	0.024	0.139	-0.225	0.152	-0.298	-0.224	-0.07	0.082	-0.319*	0.265				
Insulin	-0.286	-0.073	0.541	0.165	-0.261	-0.05	-0.241	-0.074	0.619**	-0.411	-0.499*	-0.452*	0.291	-0.473	0.515	0.856**			
BMI	0.765**	0.216	0.715**	0.605**	0.319*	0.162	0.409**	-0.226	-0.571**	-0.045	0.378**	0.464**	0.453**	0.057	0.164	0.199	-0.313		
HOMA-IR	-0.241	-0.167	0.679*	0.434	-0.107	0.087	-0.145	-0.062	0.625**	-0.402	-0.470*	-0.362	0.191	-0.333	0.396	0.714*	0.952**	-0.153	
Waist Hip ratio	0.458**	0.329*	0.253	0.866**	0.18	0.262	0.512**	-0.031	-0.455**	0.268	0.506**	0.606**	0.509**	-0.035	0.350*	0.385*	-0.363	0.341*	-0.156

Changes refer to the difference between pre- and post-intervention data. Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CRP, C-reactive protein; FT3, free triiodothyronine; FT4, free thyroxine; HBA1c, hemoglobin A1c; HDL, high-density-lipoprotein; HDL-C, high-density-lipoprotein-cholesterol; LDL, low-density lipoprotein; ; N.M. Not measured; TG, triglycerides; TSH, thyroid-stimulating hormone. *, Significant at p<0.05; ** , significant at p<0.01.

CHAPTER 2.1

The relation of saturated fatty acids with low-grade inflammation and cardiovascular disease

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Abstract

The mantra that dietary (saturated) fat must be minimized to reduce cardiovascular disease (CVD) risk has dominated nutritional guidelines for decades. Parallel to decreasing intakes of fat and saturated fatty acids (SFA), there have been increases in carbohydrate and sugar intakes, overweight, obesity and type 2 diabetes mellitus. The 'lipid hypothesis' coined the concept that fat, especially SFA, raises blood (LDL)-cholesterol and thereby CVD risk. In view of current controversies regarding their adequate intakes and effects, this review aims to summarize research regarding this heterogenic group of fatty acids and the mechanisms relating them to (chronic) systemic low-grade inflammation, insulin resistance, metabolic syndrome and notably CVD. The intimate relationship between inflammation and metabolism, including glucose, fat and cholesterol metabolism, revealed that the dyslipidemia in Western societies, notably increased triglycerides, 'small dense' LDL and 'dysfunctional' HDL, are influenced by many unfavorable lifestyle factors. Dietary SFA is only one of these, not necessarily the most important in healthy, insulin-sensitive people. The environment provides us with many other pro-inflammatory stimuli than SFA, but also with many anti-inflammatory counterparts. Resolution of the conflict between our self-designed environment and ancient genome may rather rely on returning to the pro-/anti-inflammatory balance of the Paleolithic era in consonance with the 21st century culture. Accordingly, dietary guidelines might reconsider recommendations for SFA replacement and investigate diet in a broader context, together with non-dietary lifestyle factors. This should be a clear priority, opposed to the reductionist approach of studying the effects of single nutrients, such as SFA.

Keywords

Saturated fat, coronary artery disease, cholesterol, immune system, metabolism

List of abbreviations

AHA, American Heart Association; Apo, apolipoprotein; CETP, cholesteryl ester transfer protein; CHO, carbohydrate; CLA, conjugated linoleic acid; CVD, cardiovascular disease; DM2, type 2 diabetes mellitus; DNL, *de novo* lipogenesis; energy%, percentage energy intake; FA, fatty acids; GI, glycemic index; GL, glycemic load; HDL-C, high-density lipoprotein cholesterol; HPA, hypothalamus-pituitary-adrenal; HPG, hypothalamus-pituitary-gonadal; LA, linoleic acid; LDL-C, low-density lipoprotein-cholesterol; LPS, lipopolysaccharide; MUFA, monounsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatosis; NFκB, nuclear-factor-kappa-B; PUFA, polyunsaturated fatty acids; RCTs, randomized controlled trials; SFA, saturated fatty acids; TLR, toll-like receptor, TC, total cholesterol; TG, triglyceride; VLDL, very-low density lipoprotein.

1. Introduction

The global burdens of the metabolic syndrome, and its consequences, notably type 2 diabetes mellitus (DM2) and cardiovascular disease (CVD) are alarmingly rising, producing enormous losses of life quality in both developed and developing nations [1]. Most of these burdens are preventable, since they are largely due to suboptimal lifestyle, including excessive caloric intake, unbalanced diet, physical inactivity, insufficient sleep, chronic stress, unhealthy environment (e.g. smoking) and abnormal microbial flora [2–4]. Hominins have faced major changes in both dietary and physical activity patterns and body composition since our Paleolithic ancestors emerged on Earth some 2.5 million years ago. Nowadays, there are striking differences in dietary habits and rates of chronic diseases worldwide [5] and therefore, the identification and targeting of dietary factors with the greatest potential for reducing chronic diseases, notably DM2 and CVD, are of major public health importance [6].

Fat, carbohydrates (CHO) and proteins are the primary energy-containing macronutrients consumed on a routine basis by humans. In this context, the quality, rather than the quantity, of dietary CHO and fat has become a relevant issue in the nutritional origins of cardio-metabolic conditions [6]. Among the macronutrients, fat contains the highest amount of energy per gram. A 'consumed calorie' is, however, not the same as an 'available calorie', since isocaloric diets with different macronutrient compositions have different effects on resting and total energy expenditure [7]. Total and resting energy expenditures decrease in the sequence: very low-CHO diet (CHO:-fat:protein = 10:60:30 energy%) > low-glycemic diet (40:40:20 energy%) > low-fat diet (60:20:20 energy%) [8], showing that, on an isocaloric basis, the diet with the highest protein and fat contents gives rise to the lowest weight gain.

The interest in the relation between dietary fat and CVD arose from animal studies indicating that dietary cholesterol caused arterial lesions, largely mediated through an elevation of blood cholesterol levels [9]. Since then, the relation between dietary fat and CVD risk has been intensively studied, using different approaches, including controlled feeding studies, randomized controlled trials (RCTs) and large cohort studies [10]. One of the larger studies examining CVD risk factors, the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA), already concluded, at the end of last century, that there was no clear relationship between total cholesterol (TC) and CVD [11]. Even more striking is the observation that two thirds of people admitted for acute coronary events suffer from the metabolic syndrome, but 75% of these exhibit completely normal TC and low-density lipoprotein-cholesterol (LDL-C) concentrations [12].

The 'lipid hypothesis' of CVD originated from the investigations of Ancel Keys in the nineteen fifties [13] and became exacerbated after his Seven Countries Study in the seventies [14]. Keys claimed that there was a correlation between a high dietary fat content, particularly saturated fatty acids (SFA), and both elevated serum TC and LDL-C and thereby, of CVD risk (for a historical review see [10]). Since then, dietary fat, and especially the consumption of SFA, has been consistently demonized [15]. Yet, the reduction in LDL-C from reducing SFA intake seems to be specific for large and buoyant LDL-particles, while the small and dense LDL-particles are in fact the ones implicated in CVD [16, 17]. Accordingly, the levels of small dense LDL have been shown to increase in response to low-fat-high-CHO diets [18, 19].

Most of the studies on SFA solely focused on their tendency to alter lipoprotein metabolism and to influence the concentrations of lipoproteins carrying cholesterol in blood. Therefore, the question of what constitutes the healthiest overall mixture of the different classes of dietary fats still remains unanswered. Both the agricultural and food industries are guided by recommendations to the public to decrease SFA intakes to 'as low as possible', but in dietary guidelines, no lower limit of SFA intake has been identified yet [20].

In view of the controversy regarding adequate intakes and the complex effects of fatty acids (FA), notably SFA, this review aims to summarize research findings and observations regarding this particular group of FA and the mechanisms that relate them to (chronic) systemic low-grade inflammation, insulin resistance, the metabolic syndrome, and eventually to the development of CVD, among many other typically Western diseases associated with the metabolic syndrome.

2. Saturated fatty acids. Function and occurrence

The study of lipids, and FA as their major structural elements, remains one of the most enigmatic and complex research fields in biology and nutrition. As one

Table 1. Fat and fatty acid content (saturated, mono- and poly-unsaturated) in different foods and human tissues.

	Oils					Dairy products				Meat and fish					
	Coconut oil[1]	Palm oil[1]	Fish oil (cod liver)[1]	Olive oil[1]	Sunflower oil (high LA, >65%)[1]	Butter[1]	Cheese, parmesan[1]	Milk, cow[1]	Milk, sheep[1]	Beef, tender-loin, raw[1]	Beef, sirloin, steak, raw[2]	Chicken (white meat)[2]	Game meat, antelope, raw[1]	Game meat, moose, raw[1]	Fatty fish (>10 g)
Fat (g/100g)	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**81.11**	**25.83**	**3.25**	**7.00**	**21.83**	**11.13**	**1.60**	**2.03**	**0.74**	**11.10**
Fatty acids															
SFA (g/100g FA)	**86.50**	**49.30**	**22.61**	**13.80**	**10.30**	**61.92**	**63.53**	**57.23**	**65.76**	**41.04**	**40.35**	**23.13**	**36.45**	**29.73**	**21.80**
12:0	44.60	0.10	NA	0.00	0.00	2.79	3.38	2.37	3.41	0.18	0.22	0.00	0.00	NA	0.00
14:0	16.80	1.00	3.57	0.00	0.00	10.01	11.27	9.14	9.43	3.07	3.16	0.63	0.99	0.00	4.86
16:0	8.20	43.50	10.63	11.30	5.90	26.17	26.97	25.51	23.17	23.96	24.29	16.25	16.75	12.16	15.41
18:0	2.80	4.30	2.80	2.00	4.50	12.06	8.91	11.23	12.84	12.69	12.36	6.25	18.23	17.57	8.20
PUFA (g/100g FA)	**1.80**	**9.30**	**22.54**	**10.50**	**65.70**	**3.69**	**2.20**	**6.00**	**0.31**	**3.99**	**3.80**	**25.00**	**21.67**	**32.43**	**21.71**
LA	1.80	9.10	0.94	9.80	65.70	2.25	1.05	3.69	2.59	2.57	2.44	15.00	12.32	18.92	2.43
AA	0.00	0.00	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.02	NA	0.12	0.07	NA
ALA	0.00	0.20	0.94	0.80	0.00	1.45	1.15	2.31	1.81	1.15	1.19	0.63	3.45	4.05	2.25
EPA	0.00	0.00	6.90	0.00	0.00	0.00	0.00	0.00	NA	NA	0.00	0.00	NA	NA	5.14
DHA	0.00	0.00	10.97	0.00	0.00	0.00	0.00	0.00	NA	NA	0.00	1.25	NA	NA	7.75
MUFA (g/100g FA)	**5.80**	**37.00**	**46.71**	**73.00**	**19.50**	**28.13**	**29.09**	**24.98**	**24.63**	**42.42**	**42.81**	**30.00**	**23.65**	**20.27**	**41.44**
18:1w7	5.80	36.60	NA	71.30	19.50	25.03	25.77	24.98	22.26	37.29	37.80	25.00	23.65	18.92	17.84
18:1w9			NA												

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; DHA, decosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; NA, not available; NL, The Netherlands, PUFA, polyunsaturated fatty acids; RBC, Red blood cells; SFA, saturated fatty acids; TZ, Tanzania; [1] Data from [312]; [2] Data from [313].

of the energy-producing macronutrients in our diet, fat provides essential FA and dissolves and assists in the absorption of fat-soluble vitamins and other essential nutrients. Dietary fat induces metabolic effects that are a complex consequence of the FA composition of food, its timing, and intra- and inter-individual variations. FA are key elements of all bodily tissues. They are required for several basic functions in animals, playing pivotal roles as energy sources [21], elements of membrane phospholipids [22], and as the main fuel (50%) for the production of prolonged low-intensity shivering, under cold circumstances [23]. The type of dietary fat affects vital functions of the cell and its ability to resist dysfunction, e.g. by influencing the functions of membrane-embedded receptors, enzymes and transport systems, by determining basic membrane characteristics and by producing highly active lipid mediators [24, 25].

SFA, as part of FA, are used as, among others, energy source, building blocks for structural elements, protein modification and regulation of gene transcription [21]. Compositional analyses have shown remarkable specificities for particular SFA in cellular compartments [26], though the metabolic aspects and health effects of the individual SFA are hard to examine [27]. Adipose tissue and liver own the capacity to *de novo* synthesize and store SFA, particularly palmitate (16:0), from polar precursors, notably glucose [28,29]. In addition to 16:0, the mammary gland owns the means to produce other specific SFA, such as myristic (14:0) and lauric (12:0) acids, providing a source of easily available energy, emulgating capacity and microbial protection to ensure growth, development and survival of the mammalian offspring [30].

SFA usually account for 30–40 g% of total FA in human tissues, distributed among 16:0 (15–25 g%), stearic acid (10–20 g%), 14:0 (0.5–1 g%) and 12:0 (less than 0.5 g%) [31]. Palmitic and stearic acids are universally found in natural fats, while 12:0 is especially abundant in coconut (39–54 g%) and palm kernel (44–51 g%) oils (Table 1). Major sources of 14:0 are butter fat and both coconut and palm kernel oils [27]. However, the principal dietary sources of SFA in most Western countries are full-fat dairy products, red meat and grain-based dishes (e.g. pizza, pasta and desserts) [32–34], the latter also being rich in *trans* FA, low in polyunsaturated fatty acids (PUFA) of the ω3-series and with a high glycemic load (GL), a composition remotely away from the dietary composition consumed by our Paleolithic ancestors.

Without yet entering the debate on the deleterious or beneficial effects of the different types of SFA, it has become clear that they cannot be considered as a single group in terms of structure, metabolism or cellular function [35, 36]. Our body is capable of synthesizing SFA from CHO, via *de novo* lipogenesis (DNL) [28], which are basically the same FA as present in dietary fats of animal origin, mainly 16:0, but to a lesser extent also 18:0, 14:0 and 12:0 [37]. Myristic and palmitic acids are directly involved in two classes of post-translational protein modifications, namely N-terminal myristoylation and side-chain palmitoylation [38]. The reversible attachment of 16:0 to the sulfur atom of cysteine facilitates protein-membrane interactions and the intracellular movement of proteins, and is involved in a variety of signal transduction pathways. Myristoylation includes key components in intracellular signaling pathways, oncogenes, structural viral proteins, and common constitutive eukaryotic proteins [31]. The so-called medium-chain SFA (MCFA; 6 to 12 carbons) are mainly oxidized in the liver, as e.g. demonstrated by the low fat deposition in adipose tissue from rats overfed with a medium-chain-triglyceride (TG) diet compared with rats overfed with isocaloric long-chain TGs [39].

Complex CHO, such as dietary fiber, are metabolized by the colon microbiota and then fermented to short-chain fatty acid (SCFA; less than 6 carbons), mainly acetate, propionate and butyrate [40]. The latter is used as a fuel metabolite by the colonic epithelial cells, and simultaneously prevents autophagy in these colonocytes [41]. Acetate and propionate are transported to the liver and peripheral organs, where they become substrates for gluconeogenesis and lipogenesis [40]. In addition to serving as energy sources, SCFA, notably butyrate, also affect colonic gene expression via histone deacetylase (HDAC) inhibition [42] and via metabolic regulation through signaling via G-protein-coupled receptors (GPCRs), such as GPR43. For example, SCFA have been shown to suppress inflammation through GPR43 signaling in immune cells [43] and to modulate glucagon-like peptide-1 (GLP-1) secretion, thereby improving insulin secretion [44]. Moreover, butyrate induces apoptosis in a variety of tumor cells [45] while other SFA may also influence apoptosis via the ceramide pathway through the induction of ceramide *de novo* synthesis at several steps, including serine condensation with palmitoyl–CoA [46].

3. Human milk saturated fatty acids

The FA in milk deserve special attention, since milk is the only food that is produced by the animals' own biochemical machinery and therefore provides insight into the animal's physiological needs and evolutionary past. Human milk may consequently harbor information on the importance of many nutrients, including SFA. Nevertheless, the optimal human milk composition has been subject of study for decades. There is e.g. no gold standard for the human milk FA composition. This composition is dependent on the short and long term maternal diet [47], while there is a lack of consensus regarding the optimal maternal diet [48]. The large worldwide variability of the human milk FA composition is testimony of the wide variety of foods tolerated by human beings, with dietary FA and CHO as major determinants [48]. There is, however, some degree of unity, and this is notably the case for SFA.

SFA represent around 40–60 g% of human milk FA [49], while 14:0 and shorter-chain SFA (less than 12 carbons) usually represent each about 10 g% [31] and 16:0 is about 22 g% [48] (Table 2). Despite the large biological variation in the composition of human milk FA, palmitate exhibits the lowest worldwide biological variation [48]. Myristic acid, 12:0 and shorter-chain SFA are produced in the mammary gland under the influence of a unique uncoupling protein that functions in the local *de novo* FA synthesis (DNL) from glucose. These MCFA are largely incorporated into milk TGs, following a CHO-rich meal [50]. It is, however, unlikely that, in the past, abundant dietary CHO served as a major substrate for MCFA production in the mammary gland, since the routine consumption of CHO-rich diets was not part of our culture until the start of the agricultural revolution, some 10,000 years ago [51]. The inhabitants of the island of Chole and of Dar-es-Salaam, both in Tanzania, present low CHO intakes from grains and corn, and high consumptions of coconut. Their milks exhibit the highest caprylic acid (8:0) and 12:0 contents [51] (Table 2), confirming MCFA incorporation into human milk lipids following coconut consumption [51, 52].

Milk MCFA may confer many favorable properties to the newborn. They serve as easily absorbable energy sources, and exhibit broad-spectrum antiviral and anti-microbial properties. Milk MCFA content increases with advancing lactation [30, 53], and the colostrum of mothers delivering preterm presents a higher MCFA content than those delivering at term [54].

Table 2. Fatty acid content (saturated, mono- and poly-unsaturated) in different human tissues.

Human tissues	Breast milk, Chole, TZ[1]	Breast milk, Maasai, TZ[1]	Breast milk, Jerusalem[2]	Breast milk, NL[2]	Subcutaneous adipose tissue, newborns, Curaçao,NL Antilles[3]	Subcutaneous adipose tissue, non pregnant women, Curaçao, NL Antilles[3]	RBC, newborns, Maasai, TZ[4]	RBC, adults, Maasai Wasso, TZ[5]	RBC, adults, NL[5]
Fatty acids									
SFA (g/100g FA)	**75.30**	**56.14**	**54.25**	**58.78**	**53.20**	**32.37**	**52.40**	**55.51**	**45.44**
12:0	20.17	7.82	9.67	8.20	0.30	0.58	NA	NA	NA
14:0	21.19	9.22	7.98	7.89	3.51	2.76	0.47	0.70	0.32
16:0	24.90	27.90	18.97	23.21	42.26	23.19	27.40	25.75	20.26
18:0	3.64	6.04	4.93	7.18	5.44	5.19	16.80	18.52	16.20
PUFA (g/100g FA)	**6.86**	**10.84**	**19.90**	**15.53**	**5.20**	**18.88**	**34.20**	**35.74**	**35.19**
LA	4.23	8.79	16.57	12.84	3.56	16.90	3.01	9.98	8.76
AA	0.50	0.37	0.48	0.37	0.80	0.35	16.60	13.97	14.11
ALA	0.28	0.63	0.97	1.02	0.01	0.63	0.09	0.35	0.15
EPA	0.13	0.07	0.04	0.05	0.00	0.01	0.11	0.66	0.51
DHA	0.73	0.20	0.16	0.19	0.32	0.11	4.09	2.59	4.21
MUFA (g/100g FA)	**17.85**	**33.89**	**33.18**	**33.04**	**41.60**	**48.75**	**16.60**	**18.61**	**18.73**
18:1w7	1.59	1.68	1.74	3.13	2.14	4.05	2.10	NA	NA
18:1w9	12.79	27.66	28.14	26.49	28.22	39.32	9.35	12.83	11.90

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; DHA, decosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; NA, not available; NL, The Netherlands, PUFA, polyunsaturated fatty acids; RBC, Red blood cells; SFA, saturated fatty acids; TZ, Tanzania. [1] Data from [51]; [2] Data from [48]; [3] Data from [313]; [4] Data from [314]; [5] Data from [150].

MCFA in TGs entering the neonatal `lipase. Subsequently, the pancreatic- and milk-stimulated biliary lipases can process more effectively the already partially digested TGs [55]. Because of their polarity, the released MCFA are already largely absorbed in the stomach of the neonate, therefore acting as a rapidly available energy source for the breastfed child. Lauric acid exhibits anti-microbial properties, against, among others, *Helicobacter pylori* [56] and operates synergistically with 1-monomyristyl as a bacteriostatic agent in the form of 1-monolauryl [57]. Finally, it is noteworthy that, in the developing brain, the essential FA alpha-linolenic acid is mostly converted into SFA and cholesterol [58], which are the two main culprits in the 'lipid hypothesis' of CVD [14].

Summarizing the past two paragraphs, it is clear that SFA are not essential nutrients by definition. They have, nevertheless, important functions that deserve acknowledgement. Stigmatizing SFA and removing them from our diet may not be the 'magic bullet' in the fight against the burden of typically Western diseases of affluence. The indisputably high human milk SFA content is testimony of their beneficial effects, at least in breastfed infants.

4. The 'lipid hypothesis' of Keys and its consequences

The 'lipid hypothesis' supports the concept that fat, especially SFA, and in some versions dietary cholesterol, raises blood cholesterol and thereby contributes to CVD risk [13]. However, the preliminary data from 22 different countries did not support the hypothesis that fat intake was unambiguously related with CVD (Figure 1A) [59]. Data from 15 countries were excluded (Figure 1B) in the published version of the Seven Countries Study [13]. Nevertheless, the findings gave rise to the first set of national dietary recommendations [59]. These recommendations advised to reduce total fat to 30 energy% and SFA to 10 energy%. They occurred despite the negative outcomes of RCTs published between 1965 and 1978, i.e. prior to the first issue of these recommendations in 1977 and 1983 in the USA and UK, respectively. A recent meta-analysis of these trials, investigating mortality reduction by either lower fat intake or SFA replacement by vegetable oils, revealed that serum cholesterol decreased, but not total- and CVD mortality [60].

At the end of last century, Hayes [61] highlighted the possible flaws of the equations presented by Keys and the other Seven Countries coworkers and pointed at the pivotal role of environmental and genetic variables affecting individual responses. For example, Keys et al. assumed a standard response

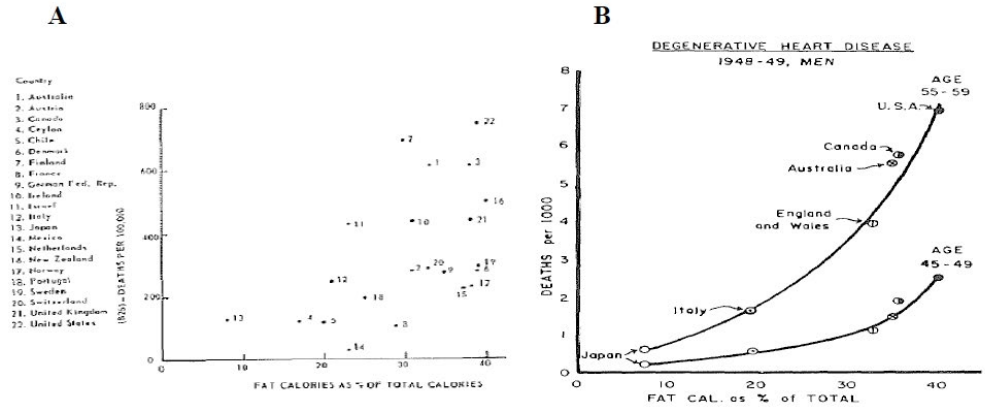

Figure 1. Data from the 'Seven Countries Study' showing mortality from arteriosclerotic and degenerative heart disease as a function of fat calories.

Panel A. Mortality from arteriosclerotic and degenerative heart disease as a function of dietary fat calories in 55-59 years old males. Data on mortality are in deaths per 100,000 inhabitants; fat calories represent the percentage of total calories. Data from [59] with permission from The Medical Society of the State of New York.

Panel B. Mortality from degenerative heart disease as a function of dietary fat calories. Data on mortality are in deaths per 1,000 inhabitants; fat calories represent the percentage of total calories. Degenerative heart diseases are categories 93 and 94 in the Revision of 1938, Categories 420 and 422 in the Revision of 1948, International List. National vital statistics are from official sources. Fat calories and percentage of total calories were calculated from National Food Balance Data of 1949 supplied by the Nutrition Division, Food and Agriculture Organization of the United Nations. Data from [13] with permission from Journal of Mount Sinai Hospital.

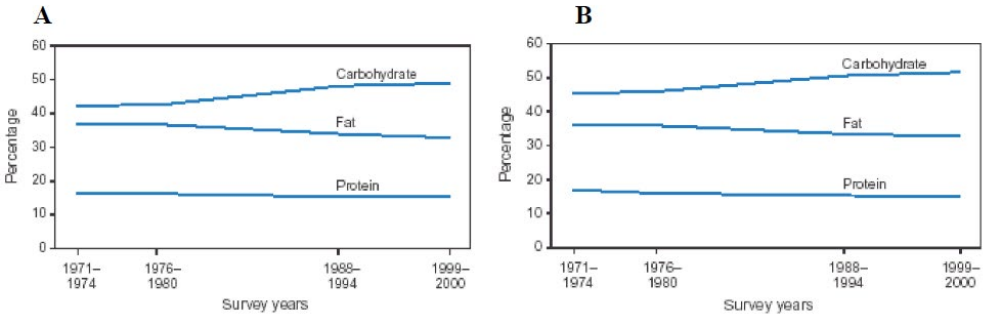

Figure 2. Energy percentages from macronutrient intake among men (panel A) and women (panel B) aged 20–74 years from 1971 to 2000 in the USA.

to dietary fat, but later studies showed that hormones and circulating lipids, among others, influence the abundance of LDL-receptors in the liver [62] and thereby plasma LDL-clearance [63]. Moreover, the presence of dietary ω3-PUFA was ignored, and all the (positive) effects caused by PUFA were granted to linoleic acid (LA) [61]. In addition, Keys did not show an association between dietary fat and mortality from all causes [59] and therefore, their data indicating that an increased fat intake increases the risk of death should not be considered as legitimate [64].

Consequently, by virtue of lack of data, the actual American Dietary Guidelines Advisory Committee Report does not provide sufficient evidence to conclude that decreases of dietary SFA, but also of e.g. salt and animal protein, would lead to positive health outcomes [65]. Nevertheless, from the nineteen seventies to 2012, CHO intake in the USA increased from 44.0 to 48.7 energy%, protein intake decreased from 16.5 to 15.7 energy% and fat from 36.6 to 33.7 energy% [66], with a concomitant reduction of SFA intake from 13.5 to about 11 energy% in men; and from 13 to

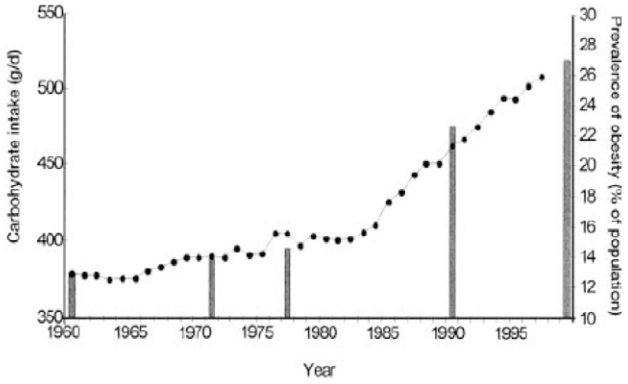

Figure 3. Carbohydrate intake and prevalence of obesity in the USA from 1960 to 1997.

11 energy% in women [67] (Figure 2). There has been a concomitant increase in the consumption of high fructose corn syrup together with a decrease in the consumption of refined sugar, to the extent that nowadays, the consumptions of these two forms of 'fast' CHO are similar [68]. In addition, in the USA and The Netherlands, as in most Western Countries, the consumption of LA is currently higher than ever along the evolution of the *homo sapiens sapiens*, and probably all of its extinct forefathers up to the common ancestor with the chimpanzee, some 6 million years ago [69–71].

From 1971–2000, mean energy intake in kilocalories increased, while energy% from carbohydrate (CHO) increased and energy% from total fat decreased. Data from the Health and Nutrition Examination Surveys (NHANES), United States, 1971–2000. For men (panel A), the energy% from CHO increased between 1971–1974 and 1999–2000 from 42.4% to 49.0% ($p<0.01$), and for women, (panel B) from 45.4% to 51.6% ($p<0.01$). The energy% from total fat decreased from 36.9% to 32.8% ($p<0.01$) for men (panel A) and from 36.1% to 32.8% ($p<0.01$) for women (panel B). The energy% from SFA decreased from 13.5% to 10.9% ($p<0.01$) for men and from 13.0% to 11.0% ($p<0.01$) for women (data not shown). A slight decrease was observed in the energy% from protein, from 16.5% to 15.5% ($p<0.01$) for men (panel A) and from 16.9% to 15.1% ($p<0.01$) for women (panel B). Sample sizes ranged from 1.730 men and 2,003 women in NHANES 1999–2000 to 6.630 men and 7.537 women in NHANES III. Adapted from the Centers of Disease and Control (CDC) [67] with permission from Centers of Disease and Control (CDC).

The mantra that (saturated) fat must be removed from the diet to reduce CVD risk has dominated both dietary advices and guidelines for decades [72]. However, parallel to the decrease in fat and SFA intake and the increase in CHO intake (particularly mono- and disaccharides) [73], there has also been an increase in the prevalence of overweight, obesity and DM2 [74–77]. In particular, since the 'diet-heart hypothesis' of Keys [13], the prevalence of obesity has increased from 11.9% in 1971 to 33.4% in 2012 (men) and from 16.6 to 36.5% (women) [74, 75] (Figure 3).

Mean carbohydrate intake (dotted line) is in g/day and obesity (BMI >30 kg/m^2, vertical bars) in % of total population. The intake of corn syrup sweeteners (as a representative for refined carbohydrates) in 1997, which were almost nonexistent at the beginning of the century, comprised 20% of the total daily carbohydrate intake and 10% of the daily total energy intake, representing an increase of 2100% in this period. The authors found a strong association between an increased consumption of refined carbohydrates in the form of corn syrup, a decreased consumption of dietary fiber, and an increasing trend in the prevalence of type 2 diabetes in the United States during the 20th century. Data from [206] with permission from The American Society for Nutrition.

5. Current intakes and recommendations: far away from a Paleolithic diet

Dietary fat is, after CHO, the main energy source in Western countries. For instance, the dietary intakes of fat in the USA [78] and the Netherlands [33] comprise about 33 and 34 energy%, respectively. The current macronutrient composition and their quality (e.g. animal/vegetable protein, fatty acid composition, complex/simple CHO) contrast with the dietary composition of our ancestors during the Paleolithic period [68] from which we do not differ much genetically, and

certainly not with respect to our main metabolic pathways. Northeast Africa is the region currently thought most pertinent to the establishment of the contemporary human genome. There, our earliest behaviorally modern ancestors of some 150,000 years ago might, have obtained, on average, about 35 energy% from fats, 35 energy% from CHO and 30 energy% from protein [79]. Nevertheless, fat intake by Paleolithic hunter-gatherers varied drastically with latitude, e.g. >60% fat in Arctic regions and <25% fat in certain tropical locations [80]. Although dietary patterns varied within, among others, latitude, season, weather, and culture, all ancestral diets shared some common key features. Food sources were limited to unprocessed plants and to foraged and hunted land and marine animals that only consumed natural foods from the local environments [68]. Traditional hunter-gatherers, unlike typically Western society members, consume all edible components of the animals they kill, including muscle meat, brain, organs, bone marrow and storage depots [81]. With the advent of both the Agricultural Revolution and animal husbandry, between 5,000 and 10,000 years ago, and more recently, the Industrial Revolution, we have dramatically altered the nutrient balance by consuming the foods typical for Western societies [82]. Game and wild plant foods contain relatively more protein, more roughage, and more micronutrients per unit of weight than the foods typically available in the current Western supermarkets [81]. In addition, while its composition varies with season, the fat of wild animals tends to have more monounsaturated fatty acids (MUFA) and PUFA and less SFA than their farm-raised counterparts [79] (Table 1).

Low-fat, high-CHO diets are within the current range of recommendations of the Institute of Medicine [83] to consume 10–35 energy% protein, 45–65 energy% CHO and 20–35 energy% fat. Such diets are however, being increasingly criticized. It was already observed in the early 1960s that very-low-fat diets (where ≤15% of total calories are derived from fat) result in hypertriglyceridemia [84]. This effect was later attributed to increased rates of hepatic DNL [29, 85] and the subsequent production of hepatic TG-rich particles, causing higher concentrations of very-low-density lipoprotein-cholesterol (VLDL-C) and lower high-density lipoprotein cholesterol (HDL-C), without yielding concomitant decreases in LDL-C [86]. Meta-analyses of RCTs did not find any potential health benefit of a low-fat diet in the general population [87, 88]. An updated review of RCTs suggested a small (14%) but potentially important reduction in CVD risk with the modification of the *type* of dietary fat, but not with the reduction of total fat [89]. As a matter of fact, short-term consumption of a low-fat diet only shows beneficial effects on plasma lipid concentrations when accompanied by weight loss [90]. A recent systematic review and meta-analysis demonstrated the beneficial effects of a high-fat vs. a low-fat diet on blood pressure and also TG, HDL-C and fasting glucose levels in both pre-diabetic subjects and patients with DM2 [91, 92].

Nowadays, partially-acculturated Greenland Eskimos obtain 4–8 energy% from SFA, but in the USA, SFA, MUFA and PUFA intakes represent about 11 [67], 12 and 7 energy% [93], respectively; and 12.5, 12.7 and 6.3 energy% in The Netherlands [33]. The estimated SFA intake by Paleolithic humans amounted to 5–7 energy%, while *trans* fats contributed in negligible amounts [79]. More recent estimates from various models studied by Kuipers et al. [70] show median Paleolithic SFA intakes ranging from 11.4 to 12.0 energy%, while the total range from all scenarios amounted to 6.8–19.0 energy%. The World Health Organization [94] and the 2010 US Dietary Guidelines [20] recommend consuming less than 10 energy% from SFA, and the American Heart Association (AHA) advices on less than 7 energy% [95], while aiming at 5–6 energy% for patients with high LDL-C [96]. The AHA also recommends ω6-PUFA intakes (i.e. LA) of 5–10 energy% [97], while the median LA intakes from our ancient diet varied from 2.3–3.6 energy% [70].

In the Netherlands, the average fish consumption hardly amounts to 3 times per month, while the recommendation is twice per week, including one portion from fatty fish. The median intakes of the sum of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids by adults amounts to 75–107 and 77–110 mg per day by women and men, respectively [33], while it is recommended to consume 450 mg/day [73] and the median intake from an East African Paleolithic diet ranged from 2.26 to 17.0 g/day, dependent on scenario [70]. Thus, in contrast to the present intakes, dietary SAFA intake was accompanied in the past by high intakes of EPA and DHA, emphasizing the importance of a balance between SAFA and PUFA, and between omega-3 and omega-6 FA, as opposed to the evaluation and recommendation of single nutrients. In addition, the median intake of vegetables and fruits by Dutch adults barely reaches 200 g per day (recommendation 400 g/day; i.e. 200 g from fruits and 200 g from vegetables) [33]. Not surprisingly, the major contributors

to diet-associated mortality in both the USA and The Netherlands were, by far, insufficient intakes of fish, vegetables and fruits, with less important roles for the intakes of saturated and *trans* FA [98].

6. (Saturated) fat and cardiovascular disease risk

Diminishing SFA consumption is recommended to reduce CVD, by virtue of its influence on lipoprotein-cholesterol levels. This chapter explores this relation and discusses the evidence linking SFA with CVD risk. It also explores the SFA-CVD risk relation in the context of nutrient replacement and embarks into the relation between CVD and the consumption of dairy and meat, the latter being the principal sources of SFA in the typical Western diet.

6. 1. Lipoproteins, fatty acids, cholesterol and CVD risk

It is almost universally accepted that high serum TC and especially LDL-C are risk factors for CVD, while a high level of HDL-C is, on the other hand, protective. Serum TC/HDL-C ratio is the consensus CVD risk factor employed for CVD risk assessment of the individual subject. In men, one unit increase in the TC/HDL-C ratio is considered to confer 53% higher CVD risk [99]. A 1% reduction in TC and LDL-C corresponds with a 2% and 1.7% CVD risk reduction, respectively [100], while an increase in HDL-C of 0.025 mmol/L (1 mg/dL) corresponds with a 2–3% decrease in CVD risk [101]. The emphasis related to SFA is usually on the seemingly unfavorable relation with LDL-C (see below). However, the causal relationship between LDL-C and CVD [102] is still subject of debate [103, 104]. There is no doubt that statins reduce TC, LDL-C and CVD risk in both primary and secondary prevention studies [105]. However, the reduction of CVD risk is accompanied by a decrease of both LDL-C and C-reactive protein [105, 106]. The pleiotropic effect of statins [107], and in particular their anti-inflammatory properties [108], may represent a solid example of the intimate relationship between (low-grade) inflammation and metabolism [3, 109].

The metabolic syndrome (later coined the 'insulin resistance syndrome' [110]) is characterized by excessive body weight, impaired glucose homeostasis, hypertension and atherogenic dyslipidemia (the 'deadly quartet'). This combination is a major risk factor for CVD and other 'typically Western' diseases [111]. The coinciding atherogenic dyslipidemia is composed of elevated TGs, small dense LDL particles and reduced HDL-C (the 'deadly lipid triad') [112]. Small dense LDL particles are susceptible to oxidation and thereby to structural modification [113]. They easily pass into the subendothelial space and may thereby promote atherosclerotic plaque formation [114] through foam cell generation, local inflammation and endothelial dysfunction [115]. Both oxidized and small dense LDL have been related to increased CVD risk [113, 116] and they occur in conjunction with the high TG and low HDL-C features of the metabolic syndrome. Under these conditions, also HDL particles change into what has unfortunately been coined 'dysfunctional HDL' because of its proinflammatory character, in contrast to 'normal HDL' ([115]; see below). Hence, it may be of more diagnostic value to employ plasma TG/HDL-C concentration ratio to define an atherogenic profile and thereby identify individuals with low insulin sensitivity and subsequent increased CVD risk [110, 117].

6.1.1. Trans fatty acids

It is universally accepted that *trans* FA increase CVD risk, most probably through their pro-inflammatory properties [118, 119]. *Trans* FA comprise industrially produced, artificial, *trans* FA (found, among others, in many fast foods, bakery products, and margarines) and naturally occurring *trans* FA (ruminant, e.g. present in dairy and meat) [120]. For instance, conjugated linoleic acid (CLA) is a *trans* FA naturally present in small amounts in ruminant fat [120]. Both observational studies on the relation of *trans* FA with risk [121, 122] and metabolic studies on the relation of *trans* FA with lipoproteins [119, 123, 124] indicated that industrial *trans* FA have detrimental effects on cardiovascular health, increase LDL-C and lower HDL-C blood levels, the latter being distinct from the effect of SFA.

A meta-analysis of observational studies showed that the replacement of 2 energy% CHO, SFA, cis-MUFA or cis-PUFA by 2 energy% industrially produced *trans* FA corresponds with 20–30% higher risk of myocardial infarction and CVD mortality [125]. Not all *trans* FA seem equal: a recent meta-analysis of prospective studies suggested that the intake of industrial *trans* FA may be positively related to CVD, while the intake from ruminant *trans* FA was not [126]. A more recent review showed that, on a weight basis, CLA, ruminant- and industrial- *trans* FA exhibited the same effect on blood lipoproteins [120]. However, the potential impact of the naturally occurring (ruminant) *trans* FA is not convincing because of the large scatter (*trans* FA

intake ranged from 2.8–10.0 g/day) and a lack of sufficient observations at high intakes [120]. Moreover, the share of natural *trans* FA in our diet is usually <0.5% [69]. Therefore, the aforementioned risks might not be applicable to the naturally occurring *trans* FA [127].

6.1.2. Mono-unsaturated and poly-unsaturated fatty acids

The substitution of 1 energy% CHO by 1 energy% PUFA, and, to a lesser extent MUFA, is accompanied by a reduction in LDL-C (means: −0.019 and −0.009 mmol/L, respectively) and TC/HDL-C ratio (−0.032 and −0.026 mol/mol) and an increase in HDL-C (0.006 and 0.008 mmol/L) [128]. Thus, based on the TC/HDL-C ratio, MUFA and PUFA appear as the healthier options, compared with CHO. On the other hand, the relation between serum lipoprotein-cholesterol concentrations and CVD risk is more complicated than until recently assumed. This has become clearly illustrated by the failures of cholesteryl ester transfer protein (CETP) inhibitors to reduce CVD risk, despite their ability to greatly increase HDL-C [129, 130] and the aforementioned pleiotropic effects of statins, which include a reduction of low-grade inflammation next to their cholesterol-lowering action [105, 131]. The intimate relationship between inflammation and metabolism [132] (further reviewed), including cholesterol metabolism [115], indicates that the changes in 'cholesterol' concentrations in Western societies are influenced by many lifestyle factors that may collectively cause low-grade inflammation and thereby CVD [2, 3]. The conflicting evidence on the association between SFA and blood lipids suggests that SFA may not be the main culprit in raising blood lipid levels, and thereby, may not be a major risk factor in the development of CVD [133].

The initial GISSI-Prevenzione [134] and JELIS [135] studies supported the use of fish oil supplements in secondary prevention of CHD, but a recent meta-analysis [136] and systematic review [137] yielded little or no effects, probably because fish oil may not contribute to a preventive effect on top of the highly effective current treatment with drugs [138]. EPA and DHA cause no change in TC and slightly increase both HDL-C and LDL-C [139, 140]. They hold important anti-arrhythmic, anti-thrombotic, anti-atherosclerotic and anti-inflammatory effects, while they also reduce serum TG and blood pressure and improve endothelial function [141]. Animal studies demonstrated that the combination of ω3-PUFA with SFA increases ω3-PUFA concentration in plasma and liver lipids [142] and that dietary SFA raise blood lipids (cholesterol and TG) only when the diet is deficient in ω3-PUFA [143]. Studies in humans showed that combining SFA and ω3-PUFA caused a synergistic beneficial effect of both types of FA, increasing red blood cell membrane fluidity, decreasing TC, LDL-C and TG, and increasing HDL-C [144, 145]. On the other hand, a high ω6-PUFA diet was shown to increase hepatic cholesterol in mice when compared with a high SFA diet [142]. The amount of ω3-PUFA required to cause any beneficial effect was maximized for mice consuming background diets with a low ω6-PUFA/SFA ratio [143]. This effect appears in line with the so-called 'Israeli paradox': the inhabitants of Israel present dietary habits low in calories, fat, SFA and high in ω6-PUFA, while their rates of Western illnesses are about the same as in the USA [146]. Altogether, these data confirm the recommendation of a diet high in ω3-PUFA with a concomitant reduction in the consumption of ω6-PUFA [133], which is basically what we have eaten in the past [147].

6.1.3. Saturated fatty acids

The harmful effect of dietary fat, notably SFA, and its relation with CVD, is increasingly questioned [69, 72, 148–151]. Recently, a revised meta-analysis of RCTs showed that the substitution of SFA (and possibly *trans* FA) by ω6-PUFA without simultaneously increasing ω3-PUFA, presents no indication of benefit and it is even likely to increase CVD risk [152] (Figure 4).

To venture into this discussion, it is important to review the historical relation between SFA intake and TC (Figure 5). This relation is subject to a strong inter-individual variation: SFA consumptions of 15 and 4% may correspond with TC values of 3 and 6 mmol/L, respectively, illustrating that SFA intake only explains a small part of TC variation. Other factors influencing the effects of SFA intake on LDL-C are the amount of dietary cholesterol, the apolipoprotein (Apo) E4 allele, obesity, insulin resistance, hypertriglyceridemia and female gender [153]. Moreover, the average TC increase due to SFA intake is low: the replacement of 1 energy% CHO by 1 energy% SFA corresponds with an LDL-C increase of 0.033–0.045 mmol/L, but also with a frequently ignored concomitant increase of HDL-C with 0.011–0.013 mmol/L [83]. These changes take place in the context of an also frequently ignored lower TG (mean −0.021 mmol/L) and without any significant effect on the TC/HDL-C ratio and

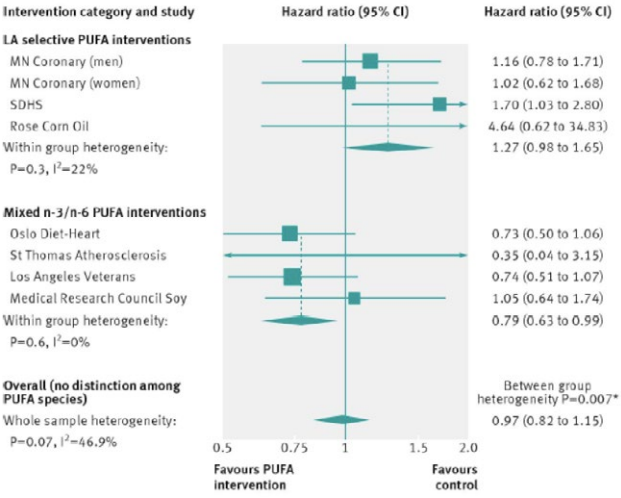

Figure 4. Meta-analysis of effects of LA selective interventions and mixed ω3/ω6 PUFA interventions on risk of cardiovascular death

LA selective interventions selectively increased intake of ω6 LA without a concurrent increase in ω-3 PUFA. Mixed PUFA interventions increased intake of ω3 PUFAs and ω6 LA. PUFA interventions replaced high SFA control diets in each trial. *Significant heterogeneity between groups. Adapted from Ramsden et al. [214] with permission from Cambridge University Press.

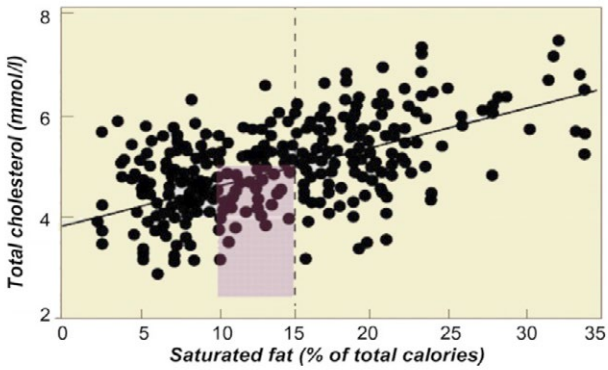

Figure 5. Relationship between energy% intake of dietary saturated fat and serum total cholesterol.

ApoB [128, 154]. For instance, in The Netherlands, a reduction of SFA intake from the current 13 energy% to the target intake of 10 energy%, would result in an irrelevant CVD risk reduction at the individual level [69], when estimated from the SCORE algorithm for CVD risk assessment. Apart from the TC/HDL-C ratio, this algorithm also takes other risk factors into account, notably gender, age, smoking and systolic blood pressure [155].

The slope amounts to 0.067 mmol/L serum cholesterol per energy% SFA (2.6 mg/dL/%). The shaded portion shows the expected benefit if saturated fat intake is lowered from 15 energy% to the currently recommended 10 energy%. This dietary change corresponds with an average serum cholesterol reduction of 0.34 mmol/L (13 mg/dL). The sizeable scatter indicates that SFA intake is an only modest determinant of total cholesterol in the population. Adapted from Volek et al. [172] with permission from Elsevier.

It has been established that adipose tissue-SFA content does not reliably reflect SFA intake and that adipose tissue-SFA is at the same time inversely related to CVD [156, 157]. Others have found that SFA-status does not correlate well with dietary SFA [158]. We [150] recently showed in an observational study of healthy African and Dutch subjects, that 14:0-, 16:0- and SFA-*status* correlate positively with the TC/HDL-C ratio. SFA-*intakes* in RCTs are, on the other hand, unrelated to this frequently used ratio for the calculation of individual CVD risk. The discrepancy between SFA-*intake* and SFA-*status* might derive from the often-overlooked endogenous SFA synthesis (DNL; notably 16:0) from CHO and other precursors, which, in contrast to the widespread believe, may

contribute to a sizeable extent to SFA-status [29, 159]. Estimations of CVD risk with the TC/HDL-C ratio as proxy suggest that the Dutch, who are representative for a population with typical Western lifestyle, would have lower CVD risk than the African populations [150]. This is highly counterintuitive, considering that more than 75% of the current adult Dutch population does not adhere to the recommendations for adults of 200 g fruits and 200 g vegetables per day, nor to the consumption of *at least* two servings of fish per week (one time fatty fish), while also their intake of CHO with high glycemic index (GI) is high [33]. These observations raise the question of whether the TC/HDL-C ratio is a suitable marker for CVD risk estimation, and underlines the usage of the aforementioned TG/HDL-C ratio as a more sensitive marker for the insulin resistance (metabolic) syndrome [110, 117].

6.2. Observational and prospective studies on saturated fat. How big is the risk?

Recent observations in a prospective cohort study of older adults, the Cardiovascular Health Study, demonstrated divergent associations of circulating 16:0 versus 18:0 and 20:0 with atrial fibrillation. Higher levels of circulating 16:0 were associated with higher risk, while higher 18:0 and 20:0 were associated with lower risk, following adjustment for other atrial fibrillation risk factors [160]. Accordingly, 18:0 does not show any deleterious effect on CVD risk [161] and the evidence for its putative effect on thrombotic susceptibility appears inconsistent [162, 163]. An update of the Nurses' Health Study showed that SFA-intake alone was not a predictor of CVD and that only a higher PUFA intake was associated with decreased CVD risk, whereas a higher *trans* FA intake was associated with increased CVD risk, independent of other dietary and CVD risk factors [164]. Furthermore, a recent study in 2,412 patients with established CVD was unable to find an association between dietary SFA intake and the incidences of both coronary events and mortality [165].

One of the strongest arguments against the presumed unfavorable effects of dietary SFA are the recent meta-analyses showing that, despite the undeniable (weak) relationship with cholesterol, dietary SFA are not associated with hard end-points [153, 166, 167], even after adjustment for serum TC [168]. Mente et al. [166] studied the outcome of prospective studies and RCTs, which analyzed the relationship between various nutritional factors and CVD. There were strong and moderate degrees of evidence found for the beneficial effects of, among others, fruit, nuts, Mediterranean diet and MUFA, fish and fish oil FA, while strong negative effects were found for *trans* FA, foods with a high GI and GL, and Western food. At the same time, there was insufficient evidence for SFA, PUFA, total fat, alpha-linolenic acid, meat, eggs and milk. Siri Tarino et al. [153] performed a meta-analysis of 21 prospective studies following 347,747 people during 5–23 years. Within this time frame, there was no relationship found between SFA intake and the development of CVD. More recently, The Health ABC Study, involving subjects with ages between 70 and 79 years, did not find any significant association between SFA consumption and CVD risk [169]. Finally, a recent study in rats has shown that virgin (highly saturated) coconut oil reduces CVD risk by exerting beneficial effects on lipid parameters through reducing DNL and enhancing the rate of FA catabolism [170].

Forsythe et al. [171] and Volek et al. [172] clearly illustrated that SFA-*intake* does not necessarily predict SFA-*status* and CVD risk (further reviewed). Their valuable data suggest that it is not only the dietary SFA content, but especially the entire dietary composition, and within this context the CHO energy%, that determines whether SFA-intake is associated with detrimental outcomes or not [150]. Populations with low CVD risk show a wide range of fat intake, varying from 15% in China to 40% in some Mediterranean populations [173], but all of them present minimal intakes of *trans* FA, high intakes of ω3-PUFA and low intakes of ω6-PUFA. Most of these low-CVD-risk populations consume low amounts of SFA, but there are a few notable exceptions with high SFA intakes. These include the Pacific Islander populations of Tokelau [174] and Kitava [175], and also the Maasai [176] and inhabitants of Chole [150] in Africa. In this context, it is noteworthy that the CVD mortality risk associated with eating too little fruit, vegetables and fish has been estimated to be 10 times greater than that due to SFA [98].

6.3. The controversy with milk and meat as saturated fat sources

Despite the sizeable contribution of both dairy products and meat to the SFA content of our diet, there is no consistent evidence that their consumption is associated with higher CVD risk [177–179]. A recent meta-analysis comparing the effects on cardio-metabolic risk markers of increasing daily intake of low-fat dairy vs. whole-fat dairy (mean 3.6 servings/day for

26 weeks) showed neither improvement nor difference between both groups [180]. In agreement with these findings, a dose-response meta-analysis of prospective studies [181] indicated that milk intake is not associated with total mortality and may even be inversely associated with overall CVD risk. This finding was confirmed in the recent Multi-Ethnic Study of Atherosclerosis (MESA) [179]. More recently, in two large prospective US cohorts (the Health Professionals Follow-Up Study and the Nurses' Health Study), circulating biomarkers of dairy fat were not associated with stroke [182], while another meta-analysis provided further evidence supporting a beneficial effect of dairy consumption on CVD [183].

Results from short-term intervention studies indicate that a diet higher in SFA from whole milk and butter increases both LDL-C and HDL-C when substituted for CHO or unsaturated FA; and might therefore not affect or even lower the TC/HDL-C ratio [184]. A study with healthy 15-year-old Swedish boys and girls found inverse associations between the dietary SFA content, mainly derived from milk, and serum concentrations of cholesterol and ApoB. These data suggest that milk fat contains, or is associated with, some component, or some other characteristics of the food intake or lifestyle, that counteracts the expected negative effect of SFA intake on serum lipids [185]. Suggestions include CLA [186] and *trans* palmitoleic acid [187]. The latter has recently been associated with higher HDL-C levels and lower TC/HDL-C ratio, adiposity, TGs, CRP, fasting insulin, blood pressure and onset of DM2 [187]. However, despite all the currently available evidence, whole fat dairy is still not recommended in most food guidelines [98, 188, 189] because of the concern that SFA in dairy food may have an adverse effect on serum lipids, increasing CVD risk.

Evidence regarding meat shows conflicting results. While the recent MESA study revealed an association between high meat intake and increased CVD risk [179], a meta-analysis of 2010 displayed no association between red meat consumption and CVD and blamed its processing for substantially increasing CVD and DM2 risks [178]. The Global Burden of Disease Study [190] identified high blood pressure, tobacco smoke and alcohol use as the three leading risk factors for disease. A diet high in red meat ranked lowest in a list of 43 risk factors contributing to the global burden of disease. Therefore, recommendations to reduce the consumption of unprocessed red meats may be unnecessarily restrictive [191]. The traditionally living Maasai, with intakes of both meat and milk that highly surpass the median Western (saturated) fat intake, present the highest 16:0 content ever measured by our group, both in RBC [150] and breast milk [51]. They have an average TC of 4.9 mmol/L [150] and almost no evidence of CVD [176, 192].

6.4. Effects of replacing saturated fat by other nutrients

Several questions regarding the relationship of SFA intake with CVD risk remain unanswered, especially whether the health effects of reducing SFA consumption are different depending on the replacement nutrient [67].

6. 4.1. Saturated fat replacement by carbohydrates

Replacing SFA with CHO with high GI values is associated with higher risk of myocardial infarction, while its replacement with CHO with low GI is associated with lower risk [193]. A recent systematic review and meta-analysis demonstrated that higher *ad libitum* CHO intakes are associated with increased TG levels [194], while exchanging either CHO or protein for fat improves the lipid-related CVD risk profile in overweight men and women [195]. Hence, the reduction of SFA intake without any consideration on the replacement nutrient may have substantial unfavorable effects on disease risk, especially because the most common replacement nutrient in populations has been CHO [154]. Prioritizing SFA replacement with CHO, notably refined CHO and added sugars, has been associated with atherogenic dyslipidemia and increased CVD risk [153]. In fact, foods containing high amounts of CHO have been implicated in the etiology of obesity and DM2 [196, 197] and are associated with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [29, 198] (further reviewed). SFA-[199] and CHO-[200] intakes are strongly associated with NAFLD in obese children, and also a low consumption of dietary fiber and ω3-PUFA [199], which underlines the importance of the multiple interacting factors playing a role in the ultimate (metabolic and inflammatory) status.

6.4.2 Low-fat-high-carbohydrate diets

Over the last years, an increasing number of study outcomes have challenged the low-fat dietary approach [92]. A diet lower in CHO and concomitantly higher in fat and protein seems a much better option

for weight loss and for the secondary prevention of chronic, typically Western, diseases [201, 202]. Liu et al. [203] suggested that the high-CHO-low-fat diet currently recommended in the USA may increase the risks of insulin resistance and glucose intolerance, and that a high dietary GL from refined CHO increases CVD risk, independent of other CVD risk factors. As an example, among subjects at high CVD risk, a Mediterranean diet (around 40 energy% fat, 40 energy% CHO and 16 energy% protein) fortified with either extra-virgin olive oil or nuts achieved a 30% improvement over a low-fat diet in terms of cardiovascular events [204]. These data are further strengthened by an RCT comparing a low-fat (with <10 energy% SFA) versus a low-CHO (12 energy% from CHO) diet [171, 172], showing that the low-CHO diet caused greater improvements on numerous endpoints such as body fatness, lipids, glucose tolerance, inflammation and thrombogenic markers (more details in paragraph 'SFA and inflammation'). Severely obese subjects (mean BMI 43 kg/m^2) with a high prevalence of DM2 or the metabolic syndrome, lost about three times more weight during six months on an *ad libitum* CHO-restricted diet (<30 g CHO/day, no calorie restriction) than on a calorie-restricted (calorie deficit, 500 kcal/day) and fat-restricted (fat intake <30 energy%) diet. Subjects following the CHO-restricted diet exhibited a relative improvement in insulin sensitivity and TG levels, even after adjustment for weight loss [205]. Increasing refined CHO intake, concomitant with decreasing intakes of fiber, paralleled the upward trend in the prevalence of both obesity and DM2 observed during the 20th [206] and 21st [75] centuries (Figure 2). Meanwhile, there is no doubt that added sugars and sugar-containing soft drinks are important determinants of blood pressure, serum lipids and body weight within the free-living population consuming an *ad libitum* diet [207, 208]. From different mechanistic points of view, high-CHO diets have been reported to reduce body fat oxidation [209] increase blood TGs [210, 211] and reduce satiety [212] compared to low-fat diets, prompting the question of their suitability for patients suffering from the metabolic syndrome, CVD and DM2 [92].

6.4.3 Saturated fat replacement by mono- and poly-unsaturated fatty acids

Evidence suggests that SFA replacement by MUFA lowers CVD risk [154], although no RCT has directly tested this relationship [213]. On the other hand, meta-analyses of RCTs have shown that partial replacement of dietary SFA for LA, the dominating dietary PUFA, is unlikely to provide the intended benefits and may actually increase the risks of both CVD and death (Figure 4) [152, 214]. An excessive ω6-PUFA intake from refined vegetable oils has been identified as a contributor to cancer and CVD [215]. In line with these data, a recent systematic review, meta-analysis and meta-regression of RCTs showed no significant benefit of SFA replacement by PUFA in the secondary prevention of CVD [216]. Another meta-analysis of 11 cohort studies and 8 RCTs where dietary SFA was replaced by PUFA [217] concluded that the benefits on CVD risk (in terms of TC/HDL-C reduction) could not distinguish between the potential benefits of increasing PUFA or reducing SFA intake. It was also noted that 'the relatively modest magnitude of plausible benefit (about 10% lower CVD risk for 5 energy% replacement) indicates a need for a substantial policy focus on other dietary risk factors for CVD, in particular high consumption of salt and low consumption of seafood, whole grains, fruits, and vegetables [217]'. These outcomes contrast with the current recommendations of the American Heart Association to limit SFA intake to <7 energy% [188] and augment LA intake to *at least* 5–10 energy% [97]. As argued by Ramsden et al. [152], this advice may not provide 'the most convincing' and 'decisive' evidence base, with 'immediate implications' for 'population and individual level recommendations' to substitute ω6-PUFA-rich vegetable oils for SFA. Finally, the arterial plaque is primarily composed of unsaturated fats, particularly PUFA (and not SFA) [215] while SFA have been suggested as the preferred fuel for the heart [218].

Taken together, recent systematic reviews and meta-analyses do not support the current guidelines encouraging a high PUFA (i.e. LA) and low SFA consumption [216, 219] and hence, dietary guidelines should strongly reconsider their recommendations for replacing SFA with ω6-PUFA [64].

7. The connection between saturated fat and inflammation

Since the discovery that obesity is associated with accumulation of macrophages in adipose tissue [220], the mechanisms by which the latter becomes inflamed, resulting in insulin resistance, have become an important research question. Several studies demonstrated that SFA might cause adipose tissue inflammation by processes involving, among others, toll-like receptor

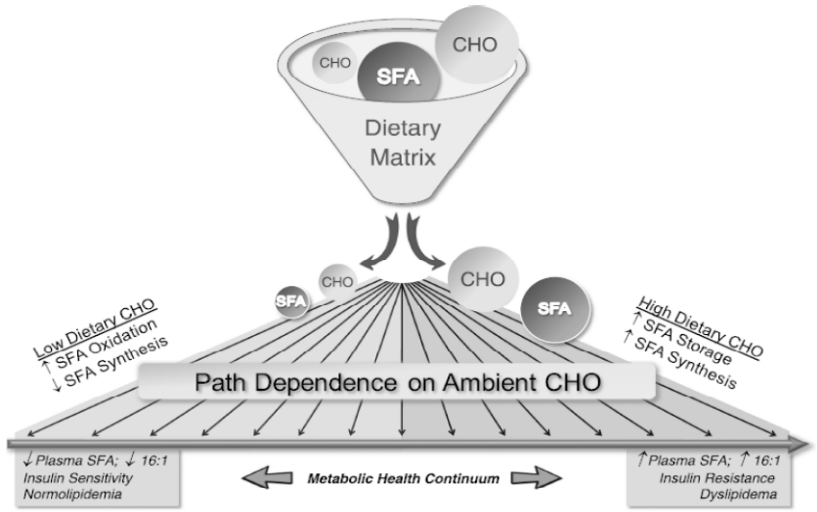

Figure 6. Importance of dietary carbohydrate in the synthesis and oxidation of saturated fat.

Right side of Figure: high-CHO diets promote the initial utilization of CHO for energy generation, causing sparing of the concomitantly consumed dietary SFA and promoting DNL (i.e. synthesis of SFA and MUFA). This occurs notably with hypercaloric CHO-rich diets, CHO with high GI, and in subjects with the metabolic syndrome (eventually causing NAFLD [29]). Concomitant DNL and sparing of dietary SFA contribute to increasing SFA status, which under such conditions becomes increasingly disconnected from dietary SFA intake, and may become deleterious when uncompensated by other factors. Left side of Figure: low dietary CHO causes oxidation of dietary SFA, low DNL and low SFA status, contributing to less SFA-induced inflammation. Adopted from [315] with permission from Wolters Kluwer Health.

(TLR) 4, a sensor that binds bacterial lipopolysaccharide (LPS) [221] and thereby plays a key role in the innate immune response [132]. This chapter embarks into the relation of SFA with inflammation.

7.1. De novo lipogenesis and insulin resistance

It has been known for at least 60 years that either fasting or dietary CHO removal results in the virtual absence of DNL [222]. The normal response to fasting, i.e. the lowering of glucose and insulin, accelerated breakdown of stored body fat and increased release of free FA (adipose tissue) and ketones (liver), is mimicked by a very-low-CHO diet [223, 224], also named ketogenic diet. Even small decreases in insulin are associated with large increases in fat breakdown and fat oxidation [225]. Conversely, due to limited storage capacity, a high CHO intake promotes the conversion of CHO to SFA (DNL), a process that becomes stimulated by pre-existing insulin resistance [29] and by the rapidity by which both glucose (high GL) and fructose enter the body [226] (Figure 6). The flux towards acetyl-CoA in the liver determines whether its origin from fat, CHO, protein or alcohol will be used only for energy generation or concomitant conversion to fat, explaining the occurrence of both alcoholic- and non-alcoholic- fatty liver disease when overloaded.

Adipose tissue acts as a buffer capable of accumulating and depositing excess lipids. Impairment of this buffering mechanism, such as in insulin resistance, results into a radical remodeling of lipid flow that promotes accumulation of lipids in the liver, skeletal muscles, and pancreatic beta cells [227]. Insulin resistance and its associated metabolic adjustments are important determinants of CVD [111]. Diets rich in fat, mainly SFA and *trans* FA, as well as CHO-rich diets, favor insulin resistance, independent of adiposity [228, 229], while dietary fiber intake is inversely associated with the risk of developing insulin resistance in adults [228].

NAFLD and its sequel, NASH, are the hepatic manifestations of the metabolic syndrome (also named the insulin resistance syndrome [110]). These conditions are among the most profound stimulators of DNL from CHO [159], while insulin resistance also stimulates fat mobilization from the adipose tissue in the form of free FA. The prevalence of NAFLD is estimated at 25–30% in the adult Western population [230]. In

patients with NAFLD, more than 25% of the FA in the liver and VLDL are *de novo* synthesized compared to less than 15% deriving from the diet [159]. In the Korean general population, the energy% from CHO is positively related to serum aminotransferase activity and metabolic syndrome prevalence, while the dietary fat percentage is inversely related [231, 232]. Other prominent sources of DNL are fructose and alcohol, which bear great resemblance with CHO in their tendency to be converted into fat in the liver [226]. However, also in this context it is the dose that makes the poison. Therefore, the total caloric intake from (other) sugars, other macronutrients [233–235], the rapidity of entry and the metabolic context of reduced insulin sensitivity should also be taken into consideration. Not surprisingly, subjects with NAFLD have been estimated to consume 5 times more CHO from soft drinks than the healthy counterparts [236].

The contribution of CHO to DNL (notably 16:0) and to a sizeable extent to SFA-status [237], was illustrated by Forsythe et al. [171], Volek et al. [172], and more recently, by Volk et al. [238]. Forsythe and Volek [171, 172] studied subjects with the metabolic syndrome and compared the effects of a high-fat, high-SFA (58.9 energy% fat, 36.4 g SFA/day), very low CHO (12.4 energy%), hypocaloric diet (1,500 kcal) versus an equally hypocaloric diet with low-fat (23.8 energy%), low-SFA (11.7 g/day) and high-CHO (55.8 energy%). They found that the high fat, high SFA (36.4 vs. 11.7 g/day), very-low-CHO hypocaloric diet did not only cause a more pronounced decrease in SFA status (in cholesterol esters and TG), but also improvements in: markers of the metabolic syndrome (greater weight loss, decreased adiposity, improved glycemic control and insulin sensitivity, and more favorable TG, HDL-C and TC/HDL-C responses); markers of 'atherogenic dyslipidemia' and CVD risk (e.g. postprandial lipidemia, apolipoproteins, LDL particle distribution, postabsorptive and postprandial vascular function); inflammatory markers (cytokines, chemokines, adhesion molecules), and markers of oxidative stress (urinary isoprostanes). In the recent study of Volk et al. [238], subjects with the metabolic syndrome were fed six different 3-week diets (300 kcal/day energy deficit) that progressively increased CHO intake (from 47 to 346 g/day) with concomitant decreases in total fat and SFA (from 84 to 32 g/day). At baseline, subjects consumed diet with 333 g CHO/day with 46 g/day SFA. CHO intake was adjusted every 3 weeks and total fat decreased proportionately to keep total energy constant. SFA intake was 40 g% of total fat for all phases. Despite a marked increase in dietary SFA intake from baseline to the first diet (46 to 84 g/day) and then a progressive decrease to 32 g/day in the last diet, the proportions of total SFA in plasma TG, CE and phospholipids of 16:0 in plasma TG and CE were not affected and were not associated with dietary CHO or SFA. Conversely, plasma palmitoleic acid, a biomarker associated with increased risk of, among others, insulin resistance, metabolic syndrome and DM2 [239] increased in all-three lipid fractions, as CHO intake increased. These data suggest that it is not only the dietary SFA content but the entire dietary composition, and especially the CHO content, that determines whether SFA intake is associated with detrimental outcomes or not. Current data indicate that when the diet is low in SFA and high in CHO, dietary SFA are spared and additionally *de novo* synthesized from the abundant dietary CHO. But when the diet is low in CHO, dietary SFA are used for energy generation.

Taken together, it has become clear that high-CHO diets promote the initial utilization of CHO for energy generation, causing sparing of the concomitantly consumed dietary SFA and promoting DNL (i.e. synthesis of SFA, notably 16:0, and MUFA, notably 18:1ω9). This situation especially occurs with hypercaloric CHO-rich diets, high CHO intake (notably CHO with high GI), high fructose and alcohol consumption, and in subjects with the metabolic syndrome [29]. Accumulating fat in the liver eventually causes NAFLD. Concomitant DNL and sparing of dietary SFA increase SFA-status, which under such conditions becomes disconnected from dietary SFA-intake. The outcome may be expected to become deleterious when uncompensated by anti-inflammatory factors and/or during circumstances of massive fat (SFA) mobilization.

7.2. SATURATED FATTY ACIDS AND INFLAMMATION

High fat diets, notably those rich in SFA [240, 241], have been shown to promote LPS (from Gram negative bacteria) uptake in the gut [242] (Figure 7). The subsequent postprandial (low-grade) endotoxemia may cause low-grade systemic inflammation [243], insulin resistance and obesity [244]. TLRs play a key role in recognizing pathogen-associated molecular patterns (PAMPs) and the subsequent activation of innate immune responses for host defense [221]. They are also crucial in shaping the adaptive immune response

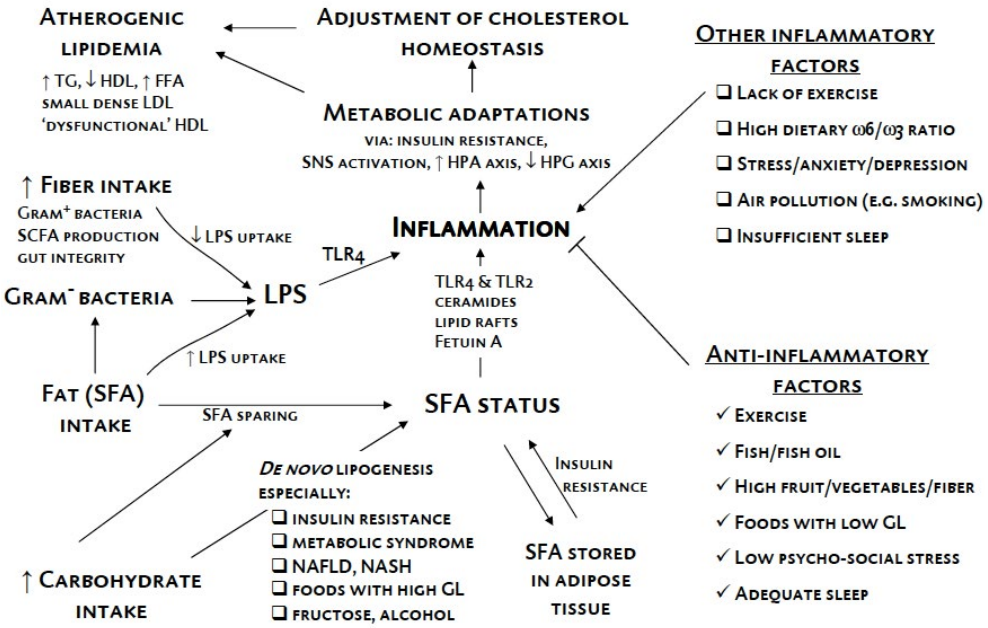

Figure 7. Factors determining SFA status and its role in chronic systemic low-grade inflammation and atherogenic dyslipidemia. Chronic systemic low-grade inflammation is central in this pathophysiological cascade. Starting in the upper left of the Figure. High fiber intake may increase Gram positive Firmicutes, resulting in a higher production of short-chain fatty acids (SCFA), which together support gut integrity and may decrease lipopolysaccharide (LPS) uptake [293, 294]. High fat diets, especially those rich in saturated fatty acid (SFA), have been shown to increase LPS uptake in the gut [241, 242]. LPS may cause inflammation by its binding to toll-like receptor 4 (TLR4) [316]. A high CHO intake induces *de novo* lipogenesis (DNL) and the production of SFA, while it also promotes sparing of dietary SFA. DNL is also stimulated by insulin resistance, the metabolic syndrome, NAFLD/non-alcoholic steatohepatitis (NASH), foods with high glycemic load (GL), fructose and alcohol [226]. A high SFA status may cause inflammation via the activation of TLR4 and TLR2, ceramide production and by the formation of lipid rafts. Fetuin A, a liver-derived circulating glycoprotein, functions as an adaptor protein directly linking SFA to TLR4 activation and promoting lipid-induced insulin resistance [269, 270]. However, fetuin A acts as a pleiotropic molecule [273], which can also promote wound healing [277]. Excessive storage of SFA in adipose tissue may cause high free SFA in insulin resistant subjects and upon fasting, and thereby contribute to inflammation. Inflammation induces adaptations in metabolic (e.g. insulin resistance) hormonal [e.g. reduced insulin sensitivity, up-regulation of the hypothalamus-pituitary-adrenal (HPA) axis, down-regulation of the hypothalamus-pituitary-gonadal (HPG) axis] and nervous pathways [e.g. sympathetic nervous system (SNS) activation], that are jointly meant for the reallocation of energy-rich nutrients that spare glucose for the brain and immune system, and forces other organs to use lipids for energy generation [3]. Among these changes, we find alterations in lipoprotein metabolism [high triglycerides (TG), high free fatty acids (FFA), low HDL] and in cholesterol homeostasis [low high-density lipoprotein (HDL), small dense low-density lipoprotein (LDL), 'dysfunctional' HDL], which are jointly known as the 'atherogenic dyslipidemia' of the metabolic syndrome. All of these inflammation-induced adaptations aim at the short-term redistribution of energy, modulation of the inflammatory reaction and the repair of the damage produced by the infectious agent and immune system. However, in the long run, they cause the typically Western diseases of the metabolic syndrome. Whether SFA plays a relevant contributing role in the development of chronic systemic low-grade inflammation (the central factor in this pathophysiological cascade) is dependent on many other pro-inflammatory factors and their balance with anti-inflammatory counterparts. Among the inflammatory factors are lack of exercise, high dietary ω6/ω3 ratio, chronic stress, anxiety and depression, air pollution (smoking included) and insufficient sleep. Anti-inflammatory factors are e.g. physical exercise, fish and fish oil, high fruits, vegetables and fiber, foods with low GL, low psycho-social stress and adequate sleep [2]. Adapted from [150] with permission from Elsevier.

from its initiation to the development of immunological memory [245], in the sensing of metabolic disturbances, and in linking immune responses to metabolic adjustments [109]. Among the different TLR subtypes, TLR-4 and TLR-2 on macrophages become activated in response to bacterial infection, tissue damage, and, according to some authors (further explained), SFA [246, 247]. LPS are among the natural ligands of the TLR-4 complex, while TLR-2 can recognize lipoproteins/lipopeptides of Gram-positive bacteria and mycoplasma [248]. LPS acts therefore as a signal molecule to detect the presence of the infectious source, with TLR-4 and TLR-2 constituting the sensors. The 'lipid A' moiety (also named endotoxin) of LPS contains 12:0 and 14:0 and their hydroxyl derivatives. These are essential for the interaction with TLR-4 [249] and abundant in coconut oil (Table 1). It is now well documented that TLRs form homo- or hetero-oligomers [250] and that the homodimerization of TLR-4 is the initial step necessary for its activation [251]. TLR-4 is located on macrophages and adipocytes, which derive from a common precursor [252]. When activated, TLRs facilitate translocation of nuclear-factor-kappa-B (NFκB) to the nucleus [253], inducing inhibition of insulin signaling, hepatic insulin resistance, activation of hepatic ceramide synthesis and other adaptations [254].

SFA can also become incorporated into specific lipid rafts domains of the plasma membrane, where they enhance TLR-4 dimerization, tyrosine-protein kinase CSK recruitment [251, 255], and the activation of downstream signaling pathways (i.e. JNK/AP-1) that may eventually inhibit insulin action [256]. Ceramides are produced via sphingomyelinase cleavage of membrane sphingolipids, from free FA by *de novo* synthesis, or by recycling of more complicated glycosylated ceramides [257]. They modulate a variety of cellular responses including cell death, autophagy, inflammation and insulin signaling [258], and can lead to insulin resistance via different mechanisms. First, they can induce dephosphorylation of AKT2, an important signaling molecule in the insulin-signaling pathway that is required for glucose transport, and thereby lower insulin signaling [259]. Second, ceramides can prevent insulin action through the activation of PKCζ, an atypical isoform of protein kinase C that binds AKT2 and thereby suppresses its activation [260] and involvement in the insulin-signaling cascade [227]. SFA are involved in *de novo* ceramide biosynthesis, where serine and palmitoyl-CoA are condensed to form 3-ketosphinganine via a synergistic signaling of TLR-4 and LPS through a non-transcriptional mechanism [261]. Erridge [262] recently showed that apparently unspoiled foodstuffs contain large quantities of TLR2- and TLR4-stimulants (i.e. a sufficient SFA-containing microbial load), and can thereby trigger inflammatory signals. In line with these findings, experimental administration of the ligands of TLR2 and TLR4, namely bacterial lipopeptides and LPS, to animal models of atherosclerosis and insulin resistance, resulted in marked amplification of both conditions [240, 263–265].

Chronic systemic low-grade tissue inflammation is emerging as a major cause of obesity-induced insulin resistance [266] with a strikingly strong accumulation of activated macrophages [267] in the adipose tissue of obese subjects [268] and strong expression of proinflammatory genes. Although TLRs appear to be a basic element of SFA-induced insulin resistance [246, 247], recent work indicates that SFA are not direct ligands for TLR-4 [269, 270] and that neither TLR-2, TLR-4 nor ceramide signaling are directly required for SFA-induced hepatic insulin resistance [271, 272]. The liver secretory glycoprotein fetuin A owns the ability to interact with a variety of receptors, including the insulin receptor and a variety of TLRs [273]. Fetuin A binds to and activates TLR-4, [269] and can also be secreted by adipose tissue, polarizing adipose tissue M2 macrophages towards the proinflammatory M1 subtype [274]. FA, like 16:0, have been demonstrated to upregulate fetuin A-mRNA expression in the liver by stimulating the binding of NFκB to the fetuin A promotor [270], inducing both inflammatory signaling and impairing insulin sensitivity [256]. Fetuin A has been presented as a biomarker of chronic inflammatory diseases, such as DM2 and atherosclerosis [270, 275]. However, recent findings have recognized fetuin A to be a pleiotropic molecule [273], as witnessed by its ability to also act as a negative acute phase reactant in the setting of sepsis and endotoxemia [276], but also to promote wound healing [277]. These apparently contradictory effects are conceivable from the logics that an inflammatory stimulus causes not only damage, but also protection from damage and stimulation of repair.

The involvement of SFA in low-grade inflammation may vary depending on the type of SFA (i.e. chain-length) [154], other dietary components, and lifestyle factors other than diet [3]. Several sources of SFA might ultimately cause inflammation via the above-mentioned pathways. SFA intake is clearly one of these, but the aforementioned DNL from a CHO-rich diet (notably with high GL), presence of

the metabolic syndrome, insulin resistance, NAFLD and high intakes of fructose and alcohol, are other sources. Insulin resistance might increase the inflammatory potential of SFA by its release from adipose tissue as free FA, pointing at the important role of (long-term) dietary habits determining adipose tissue FA composition. The intimate connection between SFA and LPS, although mechanistically yet poorly understood, reappraises the important role of the gut flora.

7.3. The role of the gut

Studies in septic patients [278] and healthy volunteers injected with low-dose LPS [279] support the concept that the systemic inflammatory response is associated with increased levels of TG and decreases in TC, HDL-C and LDL-C, together with alterations in the composition of the lipoprotein particles, such as the appearance of both small-dense LDL and the aforementioned 'dysfunctional HDL' [115]. 'Dysfunctional HDL' is proinflammatory, pro-oxidant and proatherogenic and does not support reverse cholesterol transport [280]. This newly formed HDL carries, among others, serum amyloid A instead of ApoA1, contains less esterified cholesterol, is rich in sphingolipids and rather binds to macrophages than to hepatocytes, exhibiting immunological activity. In contrast to its 'dysfunctionality', these properties are highly functional during acute inflammatory circumstances, aiding in the inflammatory reaction, while, due to the active inhibition of reverse cholesterol transport, the loss of 'normal' function causes, in an direct manner, the accumulation of cholesterol at the places where it is needed for repair [3, 115]. However, in the long run, all of the above inflammation-induced allostatic adaptations in glucose and lipid homeostasis turn into the well-known impaired glucose homeostasis and 'atherogenic dyslipidemia' components of the metabolic syndrome, which were in reality meant for the short-term energy redistribution, immune defense and repair.

As part of the host-defense mechanism against infection, the purposes of changing lipoprotein composition are the redistribution of the glucose and lipid energy sources among organs [3, 281], the prevention of an exaggerated inflammatory response by the binding of LPS [282], and also the immediate promotion of repair. LPS binds to lipid particles, which may be part of a humoral detoxification mechanism. In healthy subjects, LPS-binding protein (LBP) circulates in association with LDL, VLDL and chylomicrons, the latter being lipoproteins carrying lipids from the intestine to other tissues, and the particles with the highest LPS-inactivating capacity [283]. The association of LBP with LDL and VLDL appears to result in part from the high affinity of LBP for ApoB. Consequently, LBP plays an important role in the reduction of LPS activity by catalyzing LPS transfer from micelles to circulating lipoproteins [284]. In addition, the LBP-LPS complexes may be part of a local gut defense mechanism to fight against translocated bacterial endotoxin [21]. The enhanced concentration of circulating LBP during an acute phase response may be crucial, since it diminishes the transfer of LPS to monocytes, reduces cytokine secretion and thereby modulates the immune response [285].

An important connection between potentially pathogenic bacteria, LPS and SFA might be that the bacterial cell membrane contains important amounts of SFA and MUFA, but not PUFA [249, 286, 287]. Circulating cell wall components from Gram-negative bacteria (e.g. 'lipid A' moiety) profoundly activate TLR-2 and TLR-4, even in the central nervous system [288, 289]. In the context above, it is conceivable that SFA intake increases both LDL- and HDL-C concentrations [102, 154, 290]. We might be dealing with an evolutionary conserved immune response (further explained) to SFA and MUFA, either from bacteria or food [291], that is actually not meant to harm, but on the contrary provides a survival advantage by means of protection/prevention from bacterial overgrowth in the gut, infection, and bacterial LPS toxicity [292].

A high fiber diet, as observed in rural African populations, decreases the ratio between the Gram positive Firmicutes and the Gram negative Bacteroidetes. This causes increased production of SCFA (less than 6 carbons) [293], which in turn improves gut integrity [294] and reduces paracellular uptake of LPS. Surprisingly, a recently identified Gram negative microorganism constituting 3–5% of the gut bacterial community in healthy subjects, *Akkermansia muciniphila* [295], has been considered beneficial in the prevention of obesity and DM2. Gut colonization by *A. muciniphila* is related to decreased metabolic endotoxemia arising from a high fat diet and its activity at the mucosal surface seems to help keeping the mucosal layer in shape [295].

As Paracelsus (1493–1541) already stated, 'It is the dose (and circumstances) that makes the poison'. Our food is composed of complex biological systems, such as meat, fish, vegetables and fruits, in which the nutrients, SFA included, obey to the balance that comes

along with living material [3]. It is this balance on which hominins have evolved that may at best support our health, and it is therefore important to gain insight into the interaction of the various lifestyle factors.

7.4. Lifestyle and compensatory factors

Dietary SFA is only one of the many lifestyle factors playing a role in chronic systemic low-grade inflammation and the subsequent metabolic adaptations, including those causing changes in the concentrations of circulating lipids and lipoprotein-cholesterol. The environment provides us with many other pro-inflammatory stimuli than SFA, but also with many compensating anti-inflammatory stimuli [150] (Figure 7). Among the inflammatory factors we can find other dietary components (e.g. a high dietary ω6/ω3 ratio), but also lack of exercise, chronic stress, anxiety and depression, air pollution (e.g. smoking, fine dust) and insufficient sleep. Examples of counteracting anti-inflammatory stimuli are fish/fish oil, vegetables, fruits and fiber, low GL foods, exercise, low psychosocial stress and adequate sleep [2, 3].

Some evidence for the concept of 'compensatory factors' might come from observations of populations with traditional lifestyles. The traditionally living Maasai exhibit high intakes of SFA from milk and meat, and very low ω3-PUFA intakes from fish, if any. They might be well protected from CVD by other factors (e.g. physical fitness and a temporary total abstinence from refined CHO during warrior hood, i.e. up to age 30), resulting in capacious coronary vessels [176]. In Tanzania, it is well known that the members of the Maasai tribe become at risk of developing the features of the metabolic syndrome when they move from rural to urban areas and adopt an urban lifestyle [296]. The inhabitants of the island of Kitava are at first glance suitable candidates for high CVD risk, as they eat high quantities of CHO (69 energy%) and SFA (17 energy%) and exhibit clear metabolic signs of DNL. Nevertheless, they do not suffer from the metabolic syndrome or CVD 'despite' these seemingly unfavorable habits [297], perhaps due to their high intake of fish, high level of physical activity [297], or other compensatory anti-inflammatory factors [2, 3]. The CVD prevalence in France has been named 'paradoxical', as there is high SFA intake together with low mortality from CVD [298], an effect that has been attributed to diet quality and diversity [299], and also to the beneficial effects of moderate wine intake [300]. Non-diabetic urbanized Australian Aborigines subjected to a short-term (2 weeks) and longer-term (3 months) temporary reversion to their traditional diet (i.e. low CHO/high protein), and lifestyle in general, show an improvement in glucose tolerance and reduction of hyperinsulinemia and plasma TG concentrations [301, 302]. In addition, the major metabolic abnormalities of DM2, i.e. fasting glucose, postprandial glucose clearance, fasting plasma insulin, insulin response to glucose and plasma TGs, were either greatly improved or completely normalized in another group of Australian Aborigines after a 7 weeks reversal of the urbanization process by living as hunter-gatherers in their traditional environment [303]. At least three factors known to improve insulin sensitivity (i.e. weight loss, low-fat diet, and increased physical activity) were operating in this intervention study, and might have jointly contributed to the favorable metabolic changes observed. Moreover, different responses to modern versus historically consumed foods (i.e. foods at which humans are clearly adapted) have been demonstrated [304, 305]. As an example, a 'newly' introduced form of beef (wagyu) induced a significantly greater postprandial inflammatory response than a traditional kind of meat (kangaroo) [305]. Whilst kangaroo is native to Australia, wagyu beef is at the opposite end of the spectrum in terms of human adaptation. Nevertheless, wagyu presents high proportions of MUFA and ω-3 fatty acids relative to other beef and therefore, the difference in the inflammatory reaction may have been even greater between kangaroo and other 'newer' and more processed meats. Finally, a recent systematic review of RCTs comparing the effects of a Paleolithic nutritional pattern (CHO:fat:protein = 30:40:30 energy%) with control diets based on the current dietary guidelines (45-60:25-30:10-20 energy%) showed greater short-term improvements in waist circumference, TGs, blood pressure, HDL-C and fasting blood sugar in the Paleolithic diet group than in the control diet group in participants with one or more components of the metabolic syndrome [202].

8. The role of fat in evolution. Lipids, metabolism and immunity

Our current Western lifestyle has been proposed as the cause of many interacting false inflammatory triggers, leading to a state of chronic systemic low-grade inflammation, insulin resistance, the metabolic syndrome, and eventually to the typically Western diseases [3]. The host response to infection/inflammation comprises profound changes in lipid metabolism

and cholesterol fluxes, including increased hepatic DNL, lipolysis and cholesterol synthesis, and cholesterol redistribution [306]. Inflammation induces several metabolic adaptations via hormonal (e.g. reduced insulin sensitivity) and nervous pathways [sympathetic nervous system, hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes], which jointly lead to the reallocation of energy-rich nutrients, notably glucose and fat, but also protein [281, 307] (Figure 7). In this scenario of allostasis, lipids have been identified as part of the innate immune response, while they also play an active role in the defense against invading pathogens [308].

The above mechanisms indicate a functional link between lipid metabolism and innate immunity that employs both pathogen- and nutrient-sensing pathways. The link may trace back to an evolutionary need for survival, resulting in the simultaneous development of organ systems and signaling pathways that mediate both processes [132]. Cells involved in metabolic and immune responses show evidence of coordination and co-evolution. For instance, macrophages and adipocytes share a common lineage from mesodermal stem cells, they both carry the TLR4 [252], both secrete cytokines, and both become activated by pathogen-associated components, such as LPS [309]. As mentioned before, these evolutionary conserved mechanisms, now responsible of systemic low-grade inflammation and Western diseases, originally yielded an enormous survival advantage by protecting us from gut bacterial overgrowth and infection [291].

In view of the above, it seems logical to ponder that survival pressure would have favored both energy efficiency and storage ('thrifty genotype'), to prepare for times of food deprivation and to organize a potent immune response to defend the host against infectious agents [109]. When distressed, this system may aim at sparing glucose for the immune system and the brain via the induction of insulin resistance in selected insulin-dependent tissues and their switching to the fat burning mode, while at the same time modulating the immune response and restoring the inflicted damage [3]. However, in our current society, these metabolic adaptations, meant for survival, threat to defeat us now that we chronically respond to the products of the cultural and industrial globalization in the 21st century [310]. By constituting an allostatic load, they contribute, together with other lifestyle factors, to the pathogenesis of the metabolic syndrome and its sequels, such as atherosclerosis [115], CVD and other typically Western diseases [3]. The underlying lifestyle causes of the metabolic syndrome, and notably their persistence, have never been encountered during human evolution until (very) recently.

9. Conclusions

We content that it is the interaction between many lifestyle factors that determines whether SFA, and as a matter of fact any nutrient, contributes to systemic low-grade inflammation, changes in lipoprotein metabolism and ultimately CVD risk (Figure 7). The dysbalance between proinflammatory and anti-inflammatory stimuli in our Western society does not originate from a single cause and, consequently, may also not become solved by a single 'magic bullet'. Resolution of the conflict between our self-made environment and our ancient genome may rather rely on returning to the lifestyle of the Paleolithic era according to the culture of the 21st century [3, 68]. Accordingly, dietary guidelines might reconsider recommendations for replacing SFA, since 'food, not nutrients, is the fundamental unit in nutrition' [311]. Researching the entire diet and, even better, diet in a broader context together with non-dietary lifestyle factors, is a clear research priority, as opposed to the reductionist approach of studying the effects of single nutrients such as SFA, CHO or PUFA, even if they have been conducted in an RCT design.

10. Conflict of interest

FAJM has received grants for studying human milk nutrients, including the human milk FA composition. There are no other potential conflicts to declare.

11. Authorship

B. RN and FAJ formulated the research questions. B. RN, DAJ and FAJ designed the study. B. RN and FAJ wrote the article.

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CHAPTER 2.1a

Notes added to the SFA review in August 2017

(The relation of saturated fatty acids with low-grade inflammation and cardiovascular disease; J Nutr Biochem 2016;36:1–20)

Ancel Keys and the Seven Countries Study

Based on the recent 'White paper' of Pett et al. [1], it has come to our attention that we have mistakenly attributed Figure 1 in our saturated fatty acid (SFA) review [2] to 'The Seven Countries Study' [3], originated by Ancel Keys.

The Seven Countries Study started in 1958, to examine the relationships among lifestyle, biomarkers, and heart disease. Our Figure 1 derives from the paper of Yerushalmy and Hilleboe [4], published one year prior to the initiation of The Seven Countries Study. This paper and its figures have been used to illustrate the findings of The Seven Countries Study in the literature and social media, but they actually do not. Based on these data, Keys has been falsely accused of 'cherry picking' to make his point of a relation between SFA intake and coronary artery disease (CAD). Our Figure 1A [2] is originally from Yerushalmy and Hilleboe [4] and shows a correlation between dietary fat intake and mortality from 'arteriosclerotic and degenerative heart disease'. This Figure shows national food disappearance data from the Food and Agriculture Organization (FAO) of the United Nations, plotted against World Health Organization (WHO) data on heart disease in 22 countries. Figure 1B [2] derives originally from a paper of Keys published in 1953 [5], more than a decade before the first publications from The Seven Countries Study. It shows a similar relationship to Figure 1A, but for only six countries. Thereupon, Figures 1A and B [2] were not produced in the same study, and neither of them was from the Seven Countries Study [1]. In the below Figure we present the relation between SFA intake and 25-year coronary heart disease (CHD) mortality as genuinely derived from the Seven Countries Study, and published by Kromhout et al. in 1995 [6].

Given the inherently unreliable nature of food data and mortality prior to The Seven Countries Study [3], the question still remains of what were the criteria used by Keys for the selection of those six countries represented on the Figure from 1953 [7]. The 'White Paper' [1] explains that selection was based on 'the most reliable data' as judged by Keys but, as noted by Yerushalmy and Hilleboe [4], no justification was given for the definition of 'reliability' by Keys himself [5]. Yerushalmy and Hilleboe [4] pointed out that 'it is the responsibility of the investigator to report the basis on which the primary data are selected'. Quoting Keys, the 'White Paper' [1] notes that the data presented by Yerushalmy and Hilleboe [4] were 'not without major flaws of its own', as they used 'dietary and health data irrespective of quality', and there was also a 'chronological problem' [1]. As an example, the average diet in the mid- to late 1940s from a country is more likely to show an accurate correlation with deaths from CHD in the 1950s, than deaths in the previous decade [1].

Despite our above-mentioned omission, we do not change our opinion regarding the influence of dietary SFA on CAD. The 'White Paper' [1], however, did not aim to target this issue, explaining that it 'does not

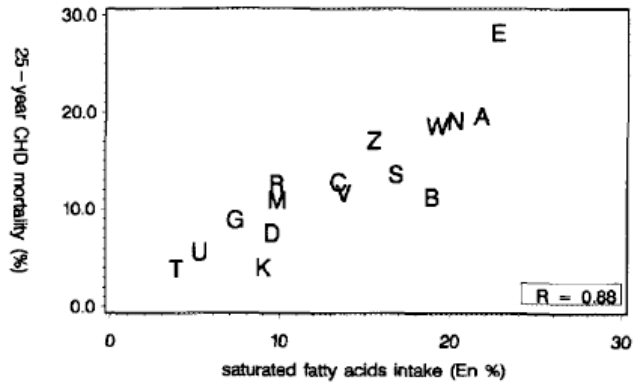

Figure. Association between average intake of saturated fat (En %) and 25-year mortality rates from coronary heart disease (CHD). A, US railroad; B, Belgrade, Serbia; C, Crevalcore, Italy; D, Dalmatia, Croatia; E, East Finland, Finland; g, Corfu, Greece; K, Crete, Greece; m, Montegiorgio, Italy; N, Zutphen, The Netherlands; R, Rome railroad, Italy; S, Slavonia, Croatia; T, Tanushimaru, Japan; U, Ushibuka, Japan; V, Velika Krsna, Serbia; W, West Finland, Finland; Z, Zrenjanin, Serbia. Adapted from [6] with permission from Elsevier.

espouse or promote any dietary advice; it is intended only to present a historically accurate account of well-documented work and redress misrepresentations of that work'. The latter refers to the work of Keys as the originator of The Seven Countries Study.

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CHAPTER 2.2

Saturated fatty acid (SFA)-status and SFA-intake exhibit different relations with serum total cholesterol and lipoprotein-cholesterol: a mechanistic explanation centered around lifestyle-induced low grade inflammation

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Abstract

We investigated the relations between fatty acid-status and serum total cholesterol, LDL-cholesterol, HDL-cholesterol and total cholesterol/HDL-cholesterol ratio in five Tanzanian ethnic groups and one Dutch group. Fatty acid-status was determined by measurement of fatty acids in serum cholesterol esters and erythrocytes. Data on the influence of fatty acid-intakes on serum total cholesterol and lipoprotein-cholesterol were obtained from documented intervention studies. The total cholesterol/HDL-cholesterol ratio is a widely used coronary artery disease (CAD) risk factor. We found that the 14:0-, 16:0- and saturated fatty acid (SFA)-status correlate positively with the total cholesterol/HDL-cholesterol ratio, while their intakes were unrelated. Linoleic acid- and polyunsaturated fatty acid (PUFA)-status and PUFA-intake exhibited negative relations with the total cholesterol/HDL-cholesterol ratio. Our data suggest that a high SFA-status is associated with increased CAD risk, while a high linoleic acid- and PUFA-status are associated with reduced CAD risk. Consequently, the total cholesterol/HDL-cholesterol ratio is a questionable risk marker, because meta-analyses of randomized controlled trials show that partial dietary replacement of SFA for linoleic acid, the dominating dietary PUFA, does not change CAD risk. We conclude that many lifestyle factors, not SFA-intake alone, determine SFA-status, and suggest that interaction with many other lifestyle factors determines whether SFA-status has a relevant contributing effect in low grade inflammation, lipoprotein changes and CAD risk. The present outcome may teach us to consider the health effects of the entire diet together with many non-dietary lifestyle factors, as opposed to the reductionist approach of studying the effects of single nutrients, SFA and PUFA included.

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Keywords

Saturated fatty acids; polyunsaturated fatty acids; fatty acid-status; fatty acid-intake; cholesterol; coronary artery disease.

List of abbreviations

ALA, alpha linolenic acid; AA, arachidonic acid; Apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CE, cholesterol esters; CETP, cholesteryl ester transfer protein; CHO, carbohydrate; DHA, docosahexaenoic acid; DNL, *de novo* lipogenesis ; EPA eicosapentaenoic acid; energy intake, en% ; FA, fatty acids; GL, glycemic load; GPR120, g protein-coupled receptor 120; HDLC, high-density lipoprotein cholesterol; HPA, hypothalamus-pituitary-adrenal; HPG, hypothalamus-pituitary-gonadal; LA, linoleic acid; LDLC, low-density lipoprotein-cholesterol; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MUFA, monounsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatosis; NFkB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; RBC, erythrocytes; SCFA, short-chain fatty acids; SFA, saturated fatty acids; SNS, sympathetic nervous system; TLR, toll-like receptor, TC, total cholesterol.

Introduction

The consumption of saturated fat (SFA) has been demonized since the finding that its intake correlates with an increase in both serum total cholesterol (TC) and low-density lipoprotein-cholesterol (LDLC), also known as the 'lipid hypothesis' (for historical review see [1]). A recent review showed that replacement of 1% energy percent (en%) carbohydrates (CHO) with 1 en% SFA, increases LDLC and high-density lipoprotein-cholesterol (HDLC) with 0.032 and 0.01 mmol/L, respectively, while it has no significant effect on the TC/HDLC ratio. LDLC is notably increased by lauric- (12:0), myristic- (14:0) and palmitic- (16:0) acids, while 12:0 also increases HDLC and lowers the TC/HDLC ratio [2]. The currently reigning paradigm is that serum TC and LDLC are risk factors for coronary artery disease (CAD) and that a high HDLC lowers CAD risk. The TC/HDLC ratio is the frequently used index for CAD risk assessment [3]. It has previously been established that, in men, 1 unit increase of the TC/HDLC ratio is associated with 53% higher risk [4].

The causality of the association between SFA and CAD is increasingly questioned [5, 6]. Dairy products and meat are the main carriers of our dietary SFA and therefore important targets for a reduced SFA intake. However, recent literature concludes there is no consistent evidence to link dairy consumption with CAD, and that even the opposite may be true [7], suggesting that dairy products may contain compensatory factors protecting from this condition [8]. A recent meta-analysis showed no association between red meat consumption and CAD, but that its processing may substantially increase CAD and diabetes risk [9]. Recent meta-analyses of prospective cohort studies [10, 11] provide little support for the association between dietary SFA and CAD, even after adjustment for serum TC [12]. It is clear that serum TC and LDLC can be lowered by diet, but this does not prove SFA to be atherogenic, since both TC and LDLC could be CAD risk markers, secondary to the real cause, notably lifestyle-induced low grade inflammation [13, 14].

Based on a meta-analysis of randomized controlled trials, the American Heart Association advocates the replacement of dietary SFA for linoleic acid (LA) to reach a SFA intake below 7 en% and a LA intake of 5–10 en% [15]. However, the choice of the underlying studies has been criticized by Ramsden et al. [16]. They found that trials with unfavorable outcomes were excluded and that in several of the included trials, large quantities of *trans* fatty acids may have been removed in addition to SFA replacement, while in some others, SFA were partially replaced by ω3-polyunsaturated fatty acids (ω3PUFA), including those present in fish oil. Moreover, in a recent reanalysis of the Sydney Diet Heart Study, this group showed that replacing dietary SFA with dietary LA increased the rates of death from all causes and CAD, while a renewed meta-analysis of the four pure SFA-to-LA randomized controlled trials showed borderline insignificant higher death from CAD (hazard ratio 1.33; 95% confidence limit 0.99–1.79) [17].

There is increasing evidence supporting a pivotal role for inflammation in all phases of atherosclerosis [18, 19]. The beneficial effects of statins were first assumed to result from their ability to reduce cholesterol synthesis and improve serum lipid profiles, but numerous pleiotropic effects of these drugs on atherosclerotic lesions have subsequently been described next to their cholesterol-lowering action, including a reduction of low grade inflammation [20, 21]. That the relation between serum lipoprotein-cholesterol concentrations and CAD risk is indeed not as simple as until recently assumed, has become strikingly illustrated by the failures of cholesteryl ester transfer protein (CETP) inhibitors to reduce CAD risk, despite their ability to greatly increase HDLC [22, 23]. An important recent finding is, however, that inflammation is intimately related to metabolism [24], including cholesterol metabolism [25], suggesting that the changes of 'cholesterol' in Western societies are secondary to many lifestyle factors that collectively cause low grade inflammation [13, 14].

We hypothesize that there are many factors interacting with dietary SFA and that the outcome of these interactions determine whether SFA will accumulate in the body, will be *de novo* produced, and will eventually have a relevant contribution to low grade inflammation or not. Whether SFA-induced inflammation is caused by SFA-intake, SFA-status, or both, is also not clear. In this context, there is surprisingly little information on the relation between SFA-status and serum total and lipoprotein cholesterol. In the present study, we investigated whether SFA-status correlates with serum TC and lipoprotein-cholesterol and whether these correlations are different from the reported effects of SFA-intake in Western societies. The study population was composed of five ethnic groups living in Tanzania and a study group selected in The Netherlands. These ethnic groups differ widely in the intakes of both SFA and LA.

Methods and materials

Study design and study groups

We included apparently healthy subjects from Tanzania [Chole (n=40), Maasai-Ruvu (n=41), Maasai-Wasso (n=63), Sengerema (n=30) and Hadzabe (n=15)] and from The Netherlands (n=30). The subjects were adult males and females consuming different types of diets. The inclusion criteria were non-pregnant and non-lactating apparently healthy adults (>16 years in Tanzania, >18 in The Netherlands). There were no exclusion criteria, except presence of disease and intake of medication. Informed consent was obtained from all subjects. Once the tribe members agreed to participate in the present study, all adults who met the inclusion criteria were included. The data on age of the subjects in Tanzania were obtained by interviews in Kiswahili. Weight and length were measured on the spot in Tanzania. The Dutch subjects self-reported their age, weight and length. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the National Institute for Medical Research in Dar-es-Salaam (NIMR/HQ/R.8a/Vol. IX/800, dated April 8, 2009).

Study group backgrounds and diets

Tanzania is inhabited by about 126 different tribes, none of which exceeds 10% of the population. Intermarriage is rare and, dependent on the directly surrounding environmental circumstances, many of the tribes have remained unique with respect to their diets. Traditionally living tribes are difficult to access and the members are often reluctant to participate [26].

The inhabitants of the island of Chole have high intakes of local marine fish and coconut, and consume plenty amounts of free fruits (oranges, mangos and bananas) and vegetables. They do not use vegetable oils for cooking and have low intakes of CHO from grains or corn. Both the Maasai Ruvu and Wasso diets consist of curdled milk and meat from their own stock, which has recently become replenished with ugali (maize porridge). Whole carcass meat consumption is a regular practice among the Maasai. Fish is usually not eaten due to traditional beliefs that it is a snake-like creature and therefore not edible, while vegetables and fruits are considered foods for cows. The people from Sengerema have a regular fish intake (average 4–5 times/week) from the nearby Lake Victoria. Ugali, muhogo (cassava root) and plantain (baked banana) are staple foods. The Hadzabe are traditional hunter-gatherers whose diet is composed of berries, roots, honey, meat and an occasional fish from the alkaline lake. They hunt small animals in the wet season and bigger game in the dry season. In recent years, corn and corn oil have inevitably become a large part of their contemporary diet [26].

Dietary fat intake in the Netherlands comprises about 34 en%, and is, after CHO, the main energy source. SFA, monounsaturated fatty acid (MUFA) and PUFA intakes are 12.5, 12.7 and 6.3 en%, respectively. Dairy products, meat and meat products, fat and cakes are the main sources of dietary fat and SFA in the Netherlands [27]. Grains, grain products and non-alcoholic beverages are the most important sources of dietary CHO. More than 75% of the current adult Dutch population does not adhere to the consumption of 200 g fruits and 200 g vegetables per day nor the consumption of at least two servings of fish per week (particularly fatty fish) [27], in adults. In fact, the average fish consumption hardly amounts to 3 times per month [27], the intake of salt is much too high [28] and so is the intake of CHO with high glycemic indices [27].

Sample collection and analyses

Blood and EDTA-anticoagulated blood were collected by venipuncture from non-fasting subjects. The samples were stored at 4°C in the dark and processed within 2 h after collection. Thrombocyte-rich plasma and RBC were separated by centrifuging the EDTA blood for 10 min at 800 *g*. The thrombocyte-rich plasma fraction was subsequently centrifuged for 10 min at 1,500 *g*. The thrombocyte poor EDTA-plasma was stored at –20°C until the analyses of the CE-FA composition.

RBC were washed three times with 0.9% NaCl. After washing, 200 µl of the RBC 50% hematocrit suspension was transferred to a teflon-sealable Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0 (internal standard). In this ready-to-trans-methylate mixture, fatty acids (FA) are stable at room temperature and in the dark for months [29].

RBC samples in methanol/HCl from Tanzania were transported to The Netherlands at room temperature for RBC-FA analyses and EDTA-plasma was transported in dry ice. Plasma CE-FA and RBC-FA compositions were determined by capillary gas chromatography/flame ionization detection in the University

Medical Center Groningen (UMCG; The Netherlands), using the previously described procedures [30]. RBC- and CE-FA contents were expressed in g/100 g (g%). Serum TC and lipoprotein-cholesterol were also measured in the UMCG, using Roche Modular (Roche, Almere, The Netherlands).

Relationship between FA-intake and TC and lipoprotein-cholesterol concentrations

To describe the influence of the intake of a specific FA on serum TC and lipoprotein-cholesterol, we selected review articles and original-research studies published in English between 1978 and 2010 through a computer-assisted literature search.

Statistics

Statistical analyses were performed with PASW version 18.0 (SPSS Inc, Chicago, IL). Kruskal-Wallis ANOVA tests followed by post-hoc tests and applying Bonferroni correction were used to determine whether there were significant between-group differences in serum TC and lipoprotein-cholesterol levels, CE-FA and RBC-FA. RBC-FA and CE-FA were correlated with serum TC and lipoprotein-cholesterol using Spearman's correlation coefficient.

Results

Characteristics, serum TC, lipoprotein-cholesterol and TC/HDLC as a CAD risk factor

Characteristics of the six study populations and their serum TC and lipoprotein-cholesterol levels are shown in Table 1. The Dutch presented the highest TC, but also the highest HDLC. Their LDLC was significantly higher compared with the Maasai-Ruvu, but their TC/HDLC ratio was lower compared with the Maasai-Wasso and Chole. When median TC/HDLC ratios were compared with the median TC/HDLC ratio of the Hadzabe adjusted to 100%, the TC/HDLC ratios of the Maasai-Ruvu, Dutch, Sengerema, Chole and Maasai-Wasso were 103, 103, 113, 128 and 131%, respectively. It was previously established by Stampfer et al. [4] that, in men, 1 unit difference in the TC/HDLC ratio confers 53% higher CAD risk. Our study comprised both men and women and, therefore, we also calculated the TC/HDLC ratio separately for men and women to find that, within each group, men and women did not differ in the TC/HDLC ratio. When considering the CAD risk deriving from the TC/HDLC ratios in ascending order, the Dutch, who may be considered as representative for subjects with a Western lifestyle, would occupy the lowest position in men and the second position in women, which is highly counterintuitive.

CE-FA and RBC-FA reflect population dietary FA composition

The CE-FA and RBC-FA compositions of the six study populations are shown in Table 1. We believe that the selected members were representative of their tribes, including dietary habits. The latter became confirmed by the observed CE-FA and RBC-FA compositions. The low intake of vegetable oils in Chole became confirmed by their lowest CE-LA and RBC-LA, while the highest CE-LA levels were found in the Dutch. Regarding the CE-LA contents, but not RBC-LA, the Hadzabe, Maasai and Sengerema held intermediate positions. The CE-14:0 and RBC-14:0 contents were highest in the subjects from Chole, most probably confirming their high coconut intake, and lowest in the Dutch and Sengerema. The highest CE-DHA and RBC-DHA contents were observed in Chole and Sengerema, confirming their high fish intake. These levels were very low in the Maasai and Hadzabe, who eat no or little fish, respectively, while the Dutch showed an intermediate position. Interestingly, the Dutch had the lowest CE-16:0, RBC-16:0, CE-SFA and RBC-SFA. The highest levels of CE-14:0 and CE-16:0 were encountered in the Chole, but they did not exhibit the highest RBC-16:0.

CE-FA and RBC-FA vs. serum TC and various lipoprotein-cholesterol contents

Table 2 shows the correlation coefficients for the associations between the CE-FA and RBC-FA contents, on the one hand, and the TC and various serum lipoprotein-cholesterol contents on the other hand. Data are given only for the whole study group composed of six populations. Similar correlations with respect of the directions (i.e. either positive or inverse) were found for the separate populations (data not shown).

Saturated fatty acids

Negative correlations were found between both CE-14:0 and RBC-14:0, and HDLC. Positive correlations were found between both CE-14:0 and RBC-14:0 and the TC/HDLC ratio. Negative correlations were present between CE-16:0 and TC and between both CE-16:0 and RBC-16:0 and HDLC. Positive correlations

Table 1. Characteristics, serum TC and lipoprotein-cholesterol, FA-status (CE-FA and RBC-FA) of the six study populations.

Anthropometrics	Hadzabe Median (range)	Maasai Wasso Median (range)	Maasai Ruvu Median (range)	Chole Median (range)	Sengerema Median (range)	Dutch Median (range)
Number	15	60	41	40	40	30
Age (years)	35 (23–55)	37 (18–60)	29.5 (17–85)	39 (16–75)	30 (19–49)	32.5 (20–62)
Gender (male/female)	11/4	22/38	9/32	18/22	0/30	11/19
Height (cm)	165 (154–174)	169 (148.5–179)	162 (148–178)	160 (150–180)	158 (149–176)	175 (162–193)
Weight (kg)	55 (47–65)	59 (35.50–100)	53 (37–89)	63 (32–95)	56 (41–80)	70 (56–100)
BMI (kg/m2)	21.37 (17.69–24.03)	20.40 (15.43–33.03)	20.28 (15.4–33.5)	23.73 (10.33–37.28)	22.81 (15.62–32.46)	22.81 (19.38–32)
Serum lipids						
Number	12	60	29	37	29	29
TC (mmol/l)	4.90 (3.80–5.60)	4.50 (2.30–7.90)	3.90 (2.60–5.50)f	4.40 (2.60–5.80)f	4.40 (2.60–5.40)f	5.10 (2.90–7.60)[c, d, e]
HDLC (mmol/l)	1.40 (1.00–1.90)[d]	1.10 (0.50–1.90)[f]	1.20 (0.40–2.00)f	1.00 (0.10–1.90)a, f	1.10 (0.60–2.10)f	1.50 (1.00–2.10)[b, c, d, e]
LDLC (mmol/l)	2.70 (2.10–3.40)	2.90 (1.40–5.40)[c]	2.20 (1.20–3.40)b, f	2.80 (1.00–4.70)	2.90 (1.10–4.10)	3.00 (1.40–5.50)[c]
TC/HDLC (mol/mol)	3.20 (2.70–5.00)	4.20 (2.30–9.80)[f]	3.30 (2.30–7.80)	4.10 (1.70–28.50)f	3.60 (2.30–7.80)	3.30 (2.30–5.30)[b, d]
Median TC/HDLC Hadzabe=100%	100	131	103	128	113	103
TC/HDLC (mol/mol) men	3.00 (2.70–5.00)[b]	4.60 (3.10–9.80)[a]	3.30 (3.00–3.40)	4.10 (2.40–6.70)	–	3.30 (2.30–5.30)
TC/HDLC (mol/mol) women	3.70 (3.10–4.70)	4.00 (2.30–6.90)	3.50 (2.30–7.80)	4.10 (1.70–10.10)	3.60 (2.30–7.80)	3.30 (2.40–5.10)
CE-fatty acids (g%)						
Number	14	63	39	40	29	16
14:0	0.60 (0.34–0.78)[b,d]	1.13 (0.54–2.43)[a, e, f]	0.94 (0.42–1.65)[d, e]	2.46 (0.50–5.02)[a, c, e, f]	0.54 (0.31–0.95)[b, c, d]	0.60 (0.30–1.19)[b,d]
16:0	13.21 (11.58–14.46)[f]	12.84 (11.36–16.27)[f]	12.78 (10.04–14.94)[f]	15.09 (12.14–19.23)[b, c, f]	13.66 (11.53–17.01)[f]	11.34 (9.86–12.53)[a, b, c, d, e]
18:0	0.95 (0.73–3.65)[b]	1.51 (0.92–7.64)[a, c, e]	1.09 (0.65–2.40)[b]	1.02 (0.67–2.45)[d]	1.08 (0.75–1.58)[b]	1.34 (0.82–1.94)
SFA	14.73 (12.83–18.88)[d]	15.41 (13.70–25.53)[d, f]	14.88 (12.5–18.05)[d]	18.88 (13.49–25.46)[c, e, f]	15.42 (13.14–19.48)[d]	13.26 (11.44–15.03)[b, d, e]
18:1 ω9	24.09 (20.14–30.84)	25.40 (18.33–40.2)[d, e, f]	22.42 (18.3–32.15)	20.86 (15.47–28.46)[b]	21.23 (16.88–29.91)[b]	18.72 (16.89–24.09)[b]
MUFA	33.40 (26.90–42.64)[f]	30.28 (21.72–49.05)[f]	27.28 (21.02–44.01)[f]	28.73 (19.39–40.94)[f]	27.17 (21.24–41.17)[f]	22.1 (19.72–27.24)[a, b, c, d, e]
LA	43.70 (33.89–47.70)[f]	45.42 (23.14–57.45)[d, f]	47.82 (32.62–55.79)[d]	36.46 (24.85–55.58)[b, c, f]	42.71 (27.95–51.08)[a, f]	53.12 (46.76–60.31)[a, b, d, e]
AA	5.73 (4.06–8.04)[d, e]	4.89 (2.10–8.50)[d, e, f]	5.71 (3.88–8.63)[d, e]	8.23 (5.29–14.84)[a, b, c]	8.21 (5.56–12.01)[a, b, c]	6.71 (4.57–9.35)[b]
ALA	0.30 (0.19–0.52)b	0.80 (0.27–2.05)[a, c, d, e]	0.53 (0.23–1.10)[b]	0.42 (0.24–1.22)[b]	0.39 (0.25–0.72)[b]	0.51 (0.29–0.76)
EPA	0.29 (0.15–0.60)[b, d, e, f]	0.73 (0.23–1.71)[a, d, e]	0.45 (0.15–0.9)[d, e]	1.08 (0.54–2.45)[a, b, c]	1.11 (0.37–2.37)[a, b, c]	0.71 (0.14–3.45)[a]
DHA	0.19 (0.12–0.43)[d, e, f]	0.24 (0.05–0.54)[d, e, f]	0.22 (0.13–0.55)[d, e, f]	1.08 (0.27–2.49)[a, b, c]	1.06 (0.36–2.03)[a, b, c]	0.55 (0.33–1.28)[a, b, c]
PUFA	51.96 (43.49–57.09)[f]	54.15 (32.44–64.58)[f]	57.21 (42.71–63.33)[f]	51.11 (40.83–65.12)	57.67 (42.44–64.78)[f]	64.66 (59.87–68.83)[a, b, c, e]
RBC-fatty acids (g%)						
Number	15	61	41	40	30	30
14:0	0.41 (0.37–1.11)[d]	0.70 (0.37–1.09)[c, e, f]	0.51 (0.23–0.76)[b, d, e]	1.00 (0.47–1.52)[a, c, e, f]	0.28 (0.19–0.38)[b, c, d]	0.32 (0.21–0.52)[b, d]
16:0	27.51 (25.04–30.13)[c, d, e, f]	25.75 (23.91–29.03)[c, e, f]	24.71 (22.17–27.02)[a, f]	25.24 (23.31–27.02)[a, f]	23.74 (22.38–26.56)[a]	20.26 (19.15–21.63)[a, b, c, d]
18:0	16.91 (15.29–21.65)	18.52 (15.73–20.71)[d, e, f]	18.15 (16.01–21.03)[d, e, f]	16.14 (13.95–17.93)	15.81 (14.44–16.97)[b, c]	16.2 (14.49–17.77)[b, c]
SFA	52.07 (50.69–58.24)[f]	55.51 (52.1–58.05)[d, e, f]	53.42 (50.57–55.05)[e, f]	51.20 (47.40–53.19)[b,f]	48.49 (46.53–50.17)[b, c]	45.44 (43.42–47.56)[a, b, c, d]
18:1ω9	12.07 (10.56–14.29)[d]	12.83 (10.81–15.93)[d, e]	12.79 (9.92–16.76)[d, e]	10.49 (8.93–12.57)[a, b, c, f]	10.76 (9.55–12.8)[b, c]	11.90 (9.76–14.25)[d]
MUFA	19.36 (16.23–21.44)[d, e]	18.61 (14.58–22.82)[d, e]	19.04 (15.58–23.29)[d, e]	16.31 (13.77–19.61)[a, b, c, f]	17.16 (14.76–19.83)[a, b, c, f]	18.73 (16.61–22.45)[d, e]
LA	10.72 (8.07–13.05)[d, e]	9.98 (5.58–14.47)[d, e]	10.18 (6.71–12.77)[d, e]	6.20 (4.11–9.95)[a, b, c, f]	7.71 (5.01–10.86)[a, b, c]	8.76 (7.01–11.26)[d]
AA	16.00 (13.24–17.75)[d, e, f]	13.97 (12.09–16.32)[c, d, e]	15.52 (12.69–17.86)[d, e, f]	12.82 (11.55–14.28)[a, b, c]	12.84 (10.94–14.94)[a, b, c]	14.11 (10.58–15.75)[a, c]
ALA	0.15 (0.11–0.31)[b]	0.35 (0.15–0.57)[a, d, e, f]	0.29 (0.16–0.46)[d, e, f]	0.10 (0.07–0.21)b, c	0.11 (0.08–0.19)[b, c]	0.15 (0.09–0.22)[b, c]
EPA	0.31 (0.17–0.51)[b, d, e, f]	0.66 (0.24–1.58)[a, c]	0.49 (0.19–0.87)[b]	0.61 (0.37–1.23)a	0.66 (0.26–1.37)[a]	0.51 (0.22–2.86)[a]
DHA	3.03 (2.07–4.21)[d, e]	2.59 (1.52–4.01)[d, e, f]	2.52 (1.65–5.08)[d, e, f]	5.93 (3.44–9.08)[a, b, c]	6.03 (3.44–8.37)[a, b, c]	4.21 (2.44–6.45)[a, b, c]
PUFA	39.45 (35.45–40.9)[b, d, e, f]	35.74 (30.68–41.02)[a, c, d]	38.29 (34.08–40.72)[b, d, e, f]	32.92 (28.91–35.53)[a, b, c, f]	34.6 (31.69–36.02)[a, c]	35.19 (33.34–37.74)[a, c, d]

Data are medians (range). Kruskal-Wallis ANOVA tests followed by post-hoc tests and applying Bonferroni correction were used to determine whether there were significant differences in serum lipids/lipoproteins and FA status (CE-FA and RBC-FA) within the six study populations. [a], significant difference with Hadzabe; [b], significant difference with Maasai-Wasso; [c], significant difference with Maasai-Ruvu; [d], significant difference with Chole; [e], significant difference with Sengerema; [f], significant difference with Dutch. Abbreviations: AA, arachidonic acid; BMI, body mass index; CAD, coronary artery disease; CE, cholesterol esters; DHA, decoxahexanoic acid; EPA, eicosapentanoic acid; HDLC, high-density lipoprotein cholesterol; LA, linoleic acid; LDLC, low-density lipoprotein cholesterol; MUFA, mono unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; RBC, red blood cells; TC, total cholesterol.

Table 2. Spearman's correlation coefficients for the relation between FA-status (CE-FA and RBC-FA) and serum TC and lipoprotein-cholesterol.

	TC		LDLC		HDLC		TC/HDLC	
Fatty acid (g%)	**CE**	**RBC**	**CE**	**RBC**	**CE**	**RBC**	**CE**	**RBC**
14:0	0.10	0.02	0.14	0.11	−0.19*	−0.19**	0.26**	0.23**
16:0	−0.19*	−0.12	−0.04	0.00	−0.45**	−0.18*	0.26**	0.15*
18:0	0.01	−0.03	0.07	0.00	−0.10	−0.07	0.13	0.07
SFA	−0.10	−0.11	0.04	0.03	−0.42**	−0.22**	0.32**	0.18*
18:1ω9	0.07	0.16*	0.07	0.06	0.11	0.21**	−0.02	−0.02
MUFA	0.09	0.17*	0.10	0.03	0.01	0.24**	0.07	−0.05
LA	−0.06	−0.08	−0.12	−0.13	0.15*	0.18*	−0.16*	−0.20**
AA	−0.12	−0.18*	−0.08	−0.25**	−0.18*	0.06	0.02	−0.16*
ALA	0.15*	0.05	0.21**	0.08	0.03	0.09	0.14	0.04
EPA	0.22**	0.28**	0.30**	0.35**	−0.07	0.03	0.22**	0.23**
DHA	0.02	0.08	0.09	0.11	−0.15*	−0.08	0.10	0.06
PUFA	−0.07	−0.13	−0.11	−0.21**	0.11	0.18*	−0.16*	−0.25**

*, significant at p<0.05; **, significant at p<0.01

Abbreviations: AA, arachidonic acid, CE, cholesterol esters; DHA, decoxahexanoic acid; EPA, eicosapentanoic acid; HDLC, high-density lipoprotein cholesterol; LA, linoleic acid; LDLC, low-density lipoprotein cholesterol; MUFA, mono unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; RBC, red blood cells; TC, total cholesterol.

were found between both CE-16:0 and RBC-16:0 and the TC/HDLC ratio. No correlations were found for CE-18:0 and RBC-18:0 with neither TC nor lipoprotein-cholesterol. Both CE-SFA and RBC-SFA showed negative correlations with HDLC and positive correlations with the TC/HDLC ratio. The latter two correlations are depicted in Figure 1, panels A and B, respectively, and show that the data of the Dutch fitted well within the data of the Tanzanian tribes, although they reside at the lower range of SFA contents. CE-SFA (panel A) explained 9.3% of the variation in TC/HDLC. For the entire group, CE-SFA ranged from 11.44–25.53 g%, which corresponds with a TC/HDLC ratio range from 3.4–5.2 mol/mol. RBC-SFA (panel B) explained 2.8% of the variation in TC/HDLC. For the entire group, it ranged from 43.42–58.24 g% corresponding with a TC/HDLC ratio ranging from 2.4–4.7 mol/mol.

Saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) in serum cholesterol esters (CE) and erythrocytes (RBC) served as parameters of SFA- and PUFA-status, respectively. The serum total cholesterol/HDL-cholesterol (TC/HDLC) ratio is the frequently used risk marker of coronary artery disease (CAD) risk [3]. In men, one unit difference in TC/HDLC ratio has been classically shown to correspond with a 53% difference in CAD risk [4]. It should be noted that our groups are composed by both men and women. The African group is composed of apparently healthy adults from five tribes living in Tanzania: Chole (n=40), Maasai-Ruvu (n=41), Maasai-Wasso (n=63), Sengerema (n=30) and Hadzabe (n=15). The Dutch group is composed of 30 apparently healthy adults. CE-SFA (panel A) ranged from 11.44–25.53 g% for the entire group, which corresponds with a TC/HDLC ratio from 3.4–5.2 mol/mol. RBC-SFA (panel B) ranged from 43.42–58.24 g% for the entire group, which corresponds with a TC/HDLC ratio from 2.4–4.7 mol/mol. CE-PUFA (panel C) ranged from 32.44–68.83 g% for the entire group, which corresponds with a TC/HDLC ratio from 5.1–3.4 mol/mol. RBC-PUFA (panel D) ranged from 28.91–41.02 g% for the entire group, which corresponds with a TC/HDLC ratio from 3.7–2.5 mol/mol.

Mono- and polyunsaturated fatty acids

RBC-18:1ω9 (oleic acid) and RBC-MUFA were positively correlated with TC and HDLC. CE-LA and RBC-LA presented a positive correlation with HDLC and a negative correlation with the TC/HDLC ratio. Negative correlations were found between RBC-AA and TC, LDLC and the TC/HDLC ratio, while CE-AA showed a negative correlation with HDLC. Positive correlations were found between CE-ALA and TC and LDLC. CE-EPA and RBC-EPA showed positive correlations with TC, LDLC and TC/HDLC ratio. A negative

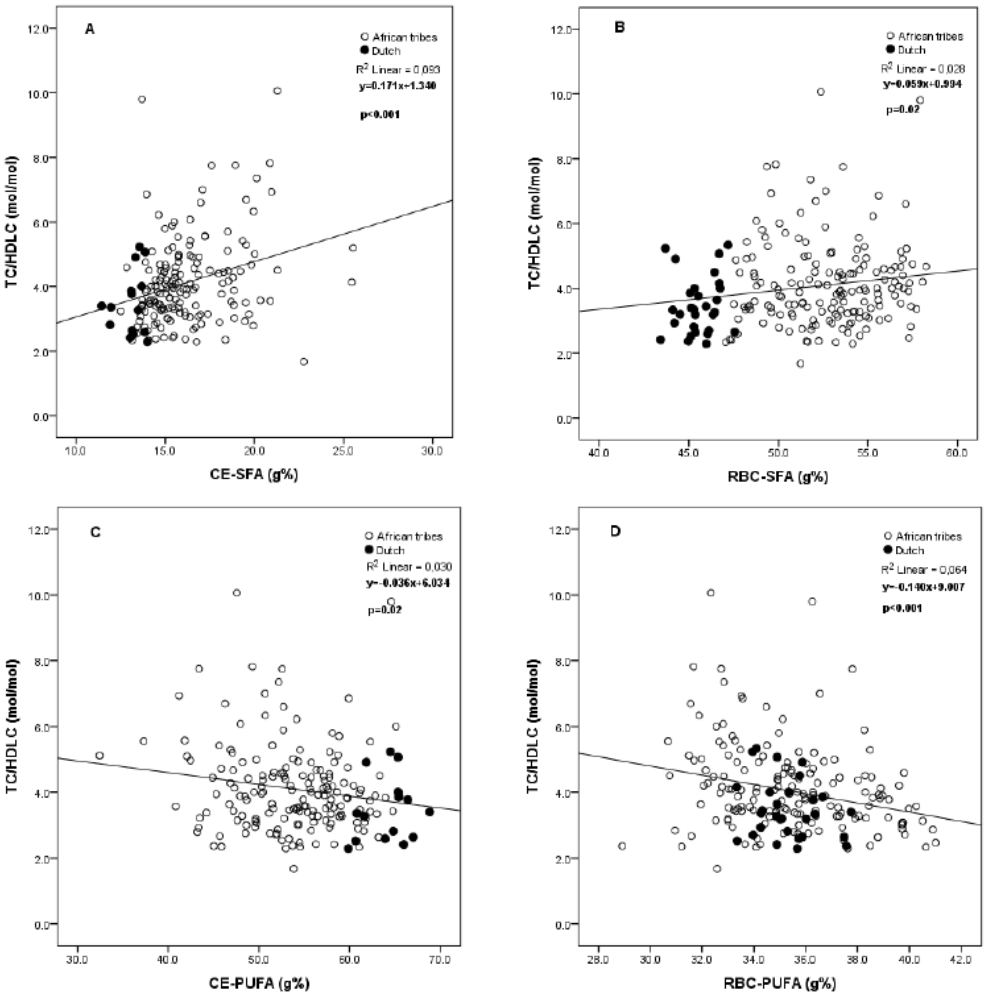

Figure 1. Relationships between the SFA- and PUFA-status and the TC/HDLC ratio

correlation was found between CE-DHA and HDLC. RBC-PUFA showed a negative correlation with LDLC and a positive relation with HDLC, while both CE-PUFA and RBC-PUFA showed negative correlations with the TC/HDLC ratio. The latter two correlations are depicted in Figure 1, panels C and D, respectively, and show that the data of the Dutch fitted well in the data of the Tanzanian tribes, although they are at the higher LA contents. CE-PUFA (panel C) explained 3.0% of the variation in the TC/HDLC ratio. For the entire group, it ranged from 32.44–68.83 g% which corresponds with a difference a TC/HDLC ratio range from 5.1–3.4 mol/mol. RBC-PUFA (panel D) explained 6.4% of the variation in TC/HDLC. For the entire group, it ranged from 28.91–41.02 g% which corresponds with a TC/HDLC ratio range from 3.7–2.5 mol/mol.

Fatty acid-status vs. serum TC and lipoprotein-cholesterol, as compared with fatty acid-intake vs. serum TC and lipoprotein-cholesterol

Table 3 shows shorthand notations for the outcomes of the correlations (either positive or inverse) of CE-FA and RBC-FA (jointly named FA-status) with serum TC and lipoprotein-cholesterol. The outcomes of these correlations were detailed in Table 2. The natures of these correlations (i.e. either positive or inverse)

Table 3. FA-status (CE-FA and RBC-FA) versus serum TC and lipoprotein-cholesterol compared with FA-intake versus serum TC and lipoprotein-cholesterol.

	TC			LDLC			HDLC			TC/HDLC		
	Status		Intake	Status		Intake	Status		Intake	Status		Intake
Fatty acid (g%)	CE	RBC		CE	RBC		CE	RBC		CE	RBC	
14:0	n.s.	n.s.	↑[1-5]	n.s.	n.s.	↑[1,3,4,5,13]	↓*	↓**	↑[1,3,4,5] n.s.[13]	↑**	↑**	n.s.[1,13]
16:0	↓*	n.s.	↑[1-5]	n.s.	n.s.	↑[1,3,4,5,13]	↓**	↓*	↑[1,3,4,5] n.s.[13]	↑**	↑*	n.s.[1,13]
18:0	n.s.	n.s.	n.s.[1,4,5,6] ↑[2] ↓[3]	n.s.	n.s.	n.s.[1,3,4,13]	n.s.	n.s.	n.s.[1,3,13] ↓[4]	n.s.	n.s.	n.s.[1,13]
SFA	n.s.	n.s.	↑[1,2,3,5,6]	n.s.	n.s.	↑[1,2,3,5,13]	↓**	↓**	↑[1,2,5,13]	↑**	↑*	n.s.[1,13]
18:1ω9	n.s.	↑*	↓[3,7]	n.s.	n.s.	↓[3,7]	n.s.	↑**	↑[3,7]	n.s.	n.s.	
MUFA	n.s.	↑*	↓[1,4] ↑[2] n.s.[6]	n.s.	n.s.	↓[1,2,4,13] n.s.[6]	n.s.	↑**	↑[1,2,4,13]	n.s.	n.s.	↓[1,13]
LA	n.s.	n.s.	↓[7]	n.s.	n.s.	↓[7]	↑*	↑*		↓*	↓**	
AA	n.s.	↓*	n.s.[10]	n.s.	↓**	n.s.[10]	↓*	n.s.	n.s.[7, 10]	n.s.	↓*	
ALA	↑*	n.s.	↓[7]	↑**	n.s.	↓[7]	n.s.	n.s.	n.s.[7]	n.s.	n.s.	
EPA	↑**	↑**	n.s.[9]	↑**	↑**	n.s.[9]	n.s.	n.s.	n.s.[9]	↑**	↑**	
DHA	n.s.	n.s.	n.s.[9, 11]	n.s.	n.s.	n.s.[9,11]	↓*	n.s.	n.s.[9] ↑[11]	n.s.	n.s.	
PUFA	n.s.	n.s.	↓[1,2,4,6,8]	n.s.	↓**	↓[1,2,4,8,13]	n.s.	↑*	↑[1,2,8,13]	↓*	↓**	↓[1,13]

Influence of FA-status (CE-FA and RBC-FA) on serum TC and lipoprotein-cholesterol, (data obtained from our study group), compared with the influence of FA-intake on serum TC and lipoprotein-cholesterol (data obtained from a literature search (see text for more information)).

↑, positive correlation; ↓, negative correlation; *, p<0.05; **, p<0.01; n.s., non significant correlation.

[1] Data from (31); [2] Data from (32); [3] Data from (33); [4] Data from (34); [5] Data from (35); [6] Data from (36); [7] Data from (37); [8] Data from (38); [9] Data from (39); [10] Data from (40); [11] Data from (41); [12] Data from (42); [13] Data from (2).

Abbreviations: AA, arachidonic acid, CE, cholesterol esters; DHA, decoxahexanoic acid; EPA, eicosapentanoic acid; HDLC, high-density lipoprotein cholesterol; LA, linoleic acid; LDLC, low-density lipoprotein cholesterol; MUFA, mono unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; RBC, red blood cells; TC, total cholesterol.

were compared with the natures of the association of FA-intakes vs. serum TC and lipoprotein-cholesterol. The latter were compiled from the literature [2,31–42].

We found many discrepancies between the correlations of the FA-status with serum TC and lipoprotein-cholesterol on one side, and with the associations of FA-intake with serum TC and lipoprotein-cholesterol on the other side. Actually, the only apparent consistencies were found in the correlations with 18:0, DHA and PUFA. Neither 18:0-status nor 18:0-intake seemed to correlate with TC, LDLC, HDLC nor TC/HDLC; while neither DHA-status nor DHA-intake correlated with TC or LDLC. On the other hand, both PUFA-status and PUFA-intake are found to correlate negatively with the TC/HDLC ratio. All other comparisons yielded inconsistencies. For instance, 16:0-status correlated negatively with HDLC, while 16:0-intake increased HDLC. 16:0-status was positively correlated with TC/HDLC ratio, but no association was found between 16:0-intake and TC/HDLC ratio. SFA-status did not correlate with TC and LDLC, but SFA-intake increased these parameters. SFA-status correlated negatively with HDLC, but SFA-intake increased HDLC. SFA-status correlated positively with TC/HDLC, but there is no effect of SFA-intake on TC/HDLC.

Focusing on the TC/HDLC ratio, the often used CAD risk factor, we found that, based on FA-status, 14:0, 16:0 and SFA seemed to have detrimental effects, while the 14:0-, 16:0- and SFA-intakes are neutral; that both 18:0-status and 18:0-intake are neutral, that the MUFA-status is neutral while the MUFA-intake is beneficial, that the LA- and PUFA-status are beneficial and so is the PUFA-intake, and finally, that the EPA-status is detrimental.

Discussion

We investigated the relation between FA-status and serum TC and lipoprotein-cholesterol in six populations with distinct races, ethnicities and lifestyles, diet included. The whole study population was composed of five ethnic groups living in Tanzania and one in The Netherlands. The groups differed widely in e.g. the SFA-intake from coconut and meat, the LA-intake from refined vegetable oils, and the intake of fish. Our main goal was to investigate whether the SFA-status of the entire study population correlates with serum TC and lipoprotein-cholesterol and whether these correlations differ from the well-known relationships between dietary SFA-intake and serum TC and lipoprotein-cholesterol, as established for Western societies. Most importantly, we found that the FA-status, as derived from the CE-FA and RBC-FA compositions, exhibits different and occasionally opposite relations with serum TC and lipoprotein-cholesterol, compared with the association between FA-intake and serum TC and lipoprotein-cholesterol. This outcome might illustrate the complexity of the relation between the dietary composition and serum TC and lipoprotein-cholesterol. Focusing on the TC/HDLC ratio, an often used CAD risk factor, we found counterintuitively higher ratios for the Maasai-Wasso and the Chole groups, when compared with the Dutch group. The 14:0-, 16:0- and SFA-status were positively correlated with the TC/HDLC ratio, while the 14:0-, 16:0- and SFA-intakes were not related with the TC/HDLC ratio. The LA- and PUFA-status and the PUFA-intake exhibited negative relations with the TC/HDLC ratio. Taken together, these findings are largely consistent with the widespread notion that SFA have hypercholesterolemic effects, while PUFA, notably LA, have hypocholesterolemic effects.

It has previously been established that adipose tissue-SFA does reflect SFA-intake and is at the same time inversely related to CAD [43, 44]. Others have found that SFA-status does not correlate well with SFA consumed in the diet [45]. The discrepancy might derive from endogenous SFA synthesis (notably 16:0) from CHO, which may contribute to a sizeable extent to SFA-status [46]. Forsythe et al. [47] and Volek et al. [48] clearly illustrated that SFA-intake does not necessarily predict SFA-status and CAD risk. They compared the effects of a high-fat, high-SFA (58.9 en% fat, 36.4 g SFA/day), very low CHO (12.4 en%), hypocaloric diet (1,500 kcal), provided to subjects with the metabolic syndrome, with those of a similar hypocaloric diet with a low-fat (23.8 en%), low-SFA (11.7 g/day) and high-CHO content (55.8 en%). They found that the high fat, high SFA (36.4 vs. 11.7 g/day), very-low-CHO hypocaloric diet, did not only cause a significantly more pronounced decrease in SFA status (in CE and triglycerides (TG)), but also improvements in: markers of the metabolic syndrome, markers associated with 'atherogenic lipidemia' and cardiovascular risk, inflammatory markers and markers of oxidative stress. Their data suggest that it is not only the dietary SFA content but the entire dietary composition, and especially the CHO content, that determines whether SFA-intake is associated

with detrimental outcomes or not. Their data also indicate that when the diet is low in SFA and high in CHO, dietary SFA are spared and additionally *de novo* synthesized from the abundant dietary CHO; while when the diet is low in CHO, dietary SFA are used for energy generation. Other prominent sources of *de novo* lipogenesis (DNL) are fructose and alcohol, which bear great resemblance in their tendency to become converted to fat in the liver [49].

A second confounder in the SFA-status vs. SFA-intake discrepancy is that conversion of CHO to SFA does not only take place at high CHO intakes, but is also stimulated by the rapidity by which both glucose (high glycemic load) and fructose enter the body, and by the existence of insulin resistance [49]. Finally, non-alcoholic fatty liver disease (NAFLD) and its sequela, non-alcoholic hepatosteatosis (NASH), are the hepatic manifestations of the metabolic syndrome (also named the insulin resistance syndrome [50]), which probably constitute the most profound conditions of *de novo* FA synthesis from CHO [51]. Among 25–30% of the adult Western population has NAFLD [52], and it has been estimated that more than 25% of the FA in the liver and very low density lipoprotein (VLDL) of patients with NAFLD are *de novo* synthesized as compared to less than 15% deriving from the diet [51]. Moreover, subjects with NAFLD consume 5 times more CHO from soft drinks than healthy persons [53], while the energy intake from CHO is positively related to serum aminotransferase activity [54].

The question of what factors are involved in the building of SFA-status and how SFA may ultimately contribute in a relevant manner to detrimental effects, might be tackled from the current view on the influence of SFA, especially 16:0, on our immune system (Figure 2). From this perspective, it is of crucial importance to appreciate that inflammation and metabolism [24], the metabolism of serum lipids/lipoprotein-cholesterol included [25], are intimately connected. Inflammation induces several adaptations in metabolism via hormonal (e.g. via the reduction of insulin sensitivity) and nervous pathways [sympathetic nervous system, hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes], which jointly lead to the reallocation of energy-rich nutrients [55, 56]. The adapted metabolism includes profound changes in both lipoprotein metabolism and cholesterol homeostasis. The latter is meant for the repair of the harm and collateral damage produced by the activity of the immune system [57, 58]. Studies in septic patients [59] and healthy volunteers [60] injected with a low dose of LPS support the concept that the systemic inflammatory response is associated with increased levels of TG due to an increase in VLDL; and decreases in TC, HDLC and LDLC, together with alterations in the composition of the lipoprotein particles, such as the appearance of small dense LDL and 'dysfunctional HDL' [25, 57]. 'Normal HDL' changes into what has been named 'dysfunctional HDL' [61, 62], which has been suggested to be proinflammatory, pro-oxidant and proatherogenic and seems to aid in the inflammatory reaction. This newly formed HDL, a.o., carries serum amyloid A (SAA) instead of apolipoprotein (apo) A1, contains less esterified cholesterol, is rich in sphingolipids and rather binds to macrophages than to hepatocytes. This loss of 'normal' function may indirectly cause the accumulation of cholesterol at the places where it is needed for repair [14, 25], due to, a.o. the active inhibition of reverse cholesterol transport. Under acute inflammatory circumstances, all these properties are highly functional, while in the long run, they turn into the well-known impaired glucose homeostasis and may eventually result in the 'atherogenic lipidemia' components of the metabolic syndrome, originally meant for the short-term energy redistribution, immune defense and repair. In turn, insulin resistance might increase the interaction of the SFA derived from the adipose tissue by increasing the free FA concentration, implying that (long-term) dietary habits determining the adipose tissue FA composition may also play a role in the exacerbation or dampening of the inflammatory reaction. The metabolic syndrome ultimately gives rise to many Western diseases, including diabetes mellitus type 2, CAD, certain forms of cancer, neurodegenerative diseases, pregnancy complications, fertility problems, and others [50], presumably because the underlying lifestyle causes of the metabolic syndrome, and notably their persistence, have never before been encountered in human evolution.

SFA, and notably long-chain SFA, have been associated with inflammation. When exposed to adipocytes *in vitro*, 16:0 causes the release of monocyte chemotactic protein-1 (MCP-1) via the TLR4-NFkB pathway, while 18:1ω9 and LA exhibit inhibitory effects [63]. Long-chain SFA have also been shown to induce apoptosis in cultured human coronary artery endothelial cells via NFkB activation [64]. How this inflammatory reaction is mechanistically accomplished is still subject of debate, but TLR4 and TLR2 [65], synthesis of ceramides [66] and the formation of lipid

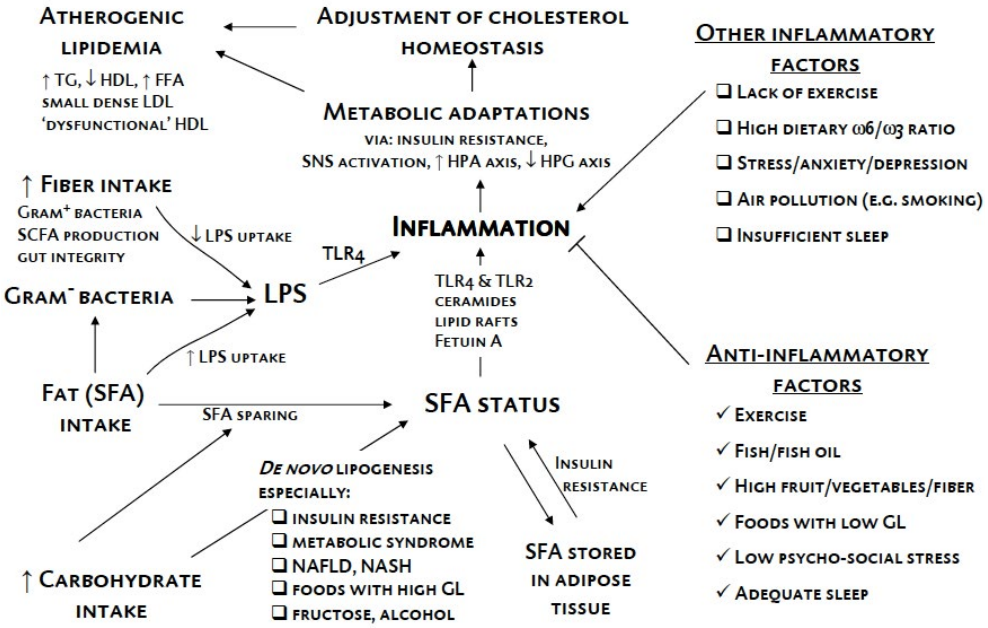

Figure 2. Factors determining SFA-status and which thereby may ultimately contribute to chronic systemic low grade inflammation and atherogenic lipidemia

A high fiber intake may increase Gram positive Firmicutes, resulting in a higher production of short-chain fatty acids (SCFA), which together support gut integrity and may decrease lipopolysaccharide (LPS) uptake [83, 84]. High fat diets, especially those rich in saturated fatty acid (SFA), have been shown to increase LPS uptake in the gut [78, 79] and LPS has been associated with inflammation via the activation the toll-like receptor 4 (TLR4) [65]. A high CHO intake induces *de novo* lipogenesis (DNL) and the production of SFA, while it also causes the sparing of dietary SFA. DNL is also stimulated by insulin resistance, the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH), high glycemic load (GL) foods, fructose and alcohol [49]. A high SFA status may cause inflammation via the activation of TLR4 and TLR2, ceramide production and by the formation of lipid rafts. More recently, fetuin A, a liver-derived circulating glycoprotein, has been shown serve as an adaptor protein that directly links SFA to TLR4 activation and promote lipid-induced insulin resistance [68, 69]. Excessive storage of SFA in adipose tissue may cause high free SFA during insulin resistance and upon fasting, and thereby contribute to inflammation. Inflammation induces adaptations in metabolic (e.g. insulin resistance) hormonal (e.g. reduced insulin sensitivity, up-regulation of the hypothalamus-pituitary-adrenal (HPA) axis, down-regulation of the hypothalamus-pituitary-gonadal (HPG) axis) and nervous pathways (e.g. sympathetic nervous system (SNS) activation), that are jointly meant for the reallocation of energy-rich nutrients that spare glucose for the brain and immune system and forces other organs to use lipids for energy generation [14]. Among these changes, we find alterations in lipoprotein metabolism (high triglycerides (TG), high free fatty acids (FFA), low HDL) and in cholesterol homeostasis (low high-density lipoprotein (HDL), small dense low-density lipoprotein (LDL), 'dysfunctional' HDL), which are jointly known as the 'atherogenic lipid profile'. All of these adaptations aim at the short-term redistribution of energy and modulation of the inflammatory reaction, and the repair of the damage produced by the immune system, but in the long run will cause the metabolic syndrome and it associated diseases. Whether SFA plays a relevant contributing role in the development of chronic systemic low grade inflammation (the central factor in this pathophysiological cascade) is dependent on many other factors that contribute to inflammation or its inhibition. Among the inflammatory factors are lack of exercise, high dietary ω6/ω3 ratio, chronic stress, anxiety and depression, air pollution (smoking included) and insufficient sleep. Anti-inflammatory factors are e.g. physical exercise, fish and fish oil, high fruits, vegetables and fiber, low GL foods, low psycho-social stress and adequate sleep [13].

rafts [67] have been implicated. More recently, fetuin A, a liver-derived circulating glycoprotein, has been shown to serve as an adaptor protein that directly links SFA to TLR4 activation and promotes lipid-induced insulin resistance [68, 69], while other *in vivo* studies suggest that insulin resistance is independent of

the TLR-4–ceramide pathway [70]. On the other hand, medium-chain SFA have shown beneficial health effects including suppression of body fat accumulation and obesity [71, 72]. PUFA, notably fish oil FA, may inhibit inflammation, and thereby counteract the inflammatory action of SFA, by inhibiting the various inflammatory pathways [73, 74], e.g. by their interaction with peroxisome proliferator-activated receptors (PPARs) [58, 74], and g protein-coupled receptor 120 (GPR120) [74, 75] and by modulation of lipid rafts [67, 74]. Alcock et al. [76] have proposed that the immune system has developed the ability to react to dietary FA as an early warning system that detects threatening changes in infectious risk at the microbial-epithelial interface. Commonly consumed FA enhancing colonization and growth of pathogens and pathobionts (like SFA) would have direct pro-inflammatory effects, while dietary FA suppressing this upgrowth (like unsaturated FA) would have direct anti-inflammatory effects.

Several sources of SFA might ultimately cause inflammation via the above mentioned pathways. SFA-intake is clearly one of these, but the aforementioned DNL from a CHO-rich diet (notably with a high glycemic load), presence of the metabolic syndrome, insulin resistance, and NAFLD and high intakes of fructose and alcohol are other sources. Insulin resistance might increase the interaction of the SFA that derive from the adipose tissue by increasing the free FA concentration, implying that (long-term) dietary habits determining the adipose tissue FA composition may also play a role in the exacerbation or dampening of the inflammatory reaction.

Another explanation for the discrepancy of the effects of SFA-intake and SFA-status on inflammation and serum TC and lipoprotein-cholesterol might ensue from the influence of a high fat concentration in the gut. High fat diets, notably those rich in SFA [77, 78], have been shown to promote lipopolysaccharide (LPS; from Gram negative bacteria) uptake in the gut [79, 80]. Moreover, it has been shown that dietary fat augments circulating LPS concentrations and that the resultant postprandial endotoxemia may cause low grade systemic inflammation [81], and initiate obesity and insulin resistance [80, 82]. A high fiber diet, as observed in rural African populations, decreases the ratio between the Gram positive Firmicutes and the Gram negative Bacteroidetes. This causes increasing short-chain fatty acid (SCFA) production [83], which in turn improves gut integrity [84] and reduces paracellular LPS uptake.

In the end, SFA-status might only be one of the many factors contributing to chronic systemic low grade inflammation and the subsequent metabolic adjustments, including those in circulating lipids and lipoprotein-cholesterol, while there may also be many compensatory anti-inflammatory stimuli counteracting the inflammatory stimuli of SFA, notably 16:0. Among the inflammatory stimuli we can find lack of exercise, a high dietary ω6/ω3 ratio, chronic stress, anxiety and depression, air pollution and insufficient sleep. Examples of counteracting anti-inflammatory stimuli are exercise, fish/fish oil, vegetables, fruits and fiber, low GL foods, low psychosocial stress and adequate sleep [13, 14]. Examples of these 'compensatory factors' might derive from observations in populations with traditional lifestyles. The traditionally living Maasai have high SFA intakes from milk and meat, and low ω3 intakes from fish. Studies in the past have shown they do not die of CAD, even though they exhibit extensive atherosclerotic lesions that are different from the ones encountered in Western societies [85]. It seems that they are protected from CAD by other factors (e.g. physical fitness and a temporary total abstinence from refined CHO during warriorhood, i.e. up to age 30), which causes their coronary vessels to be capacious [85]. Another example are the inhabitants of the island of Kitava, who at first glance should be suitable candidates for a high CAD risk, as they eat a high amount of CHO (69 en%) and SFA (17 en%) and exhibit clear metabolic signs of DNL. Despite these habits, they do not suffer from the metabolic syndrome or CAD [86], perhaps due to their high fish intake, high level of physical activity [86], or other possible compensatory anti-inflammatory factors [13].

This study has many limitations. An important one is the pooling of data from subpopulations differing in race, environmental factors and cultural habits, diet included. However, we argue that, if there is an important causal relationship between SFA and serum TC and lipoprotein-cholesterol, it should be noticeable under various confounding genetic and environmental conditions. In line with this notion, we found that the relation between SFA and the TC/HDLC ratio of the Dutch fitted well into the data of the Tanzanian tribes (Figure 1). Another limitation is that the information on the relationship between FA-intake and serum TC and lipoprotein-cholesterol is derived from intervention studies where SFA replaced CHO, while our study is purely observational.

However, we contend that the encountered discrepancy between FA-status and FA-intake should be viewed as a support for the thesis that interrelationships among single nutrients (SFA, in this paper), serum TC and lipoprotein-cholesterol and disease, may be difficult to comprehend because of the many confounding factors involved. Considering the many lifestyle factors and the many possible combinations of these factors (Figure 2), it cannot be excluded that populations with different compensatory factors may exhibit different relationships between SFA-status and serum TC and lipoprotein-cholesterol than those in the present study, while other populations may exhibit different associations between SFA-intake and serum TC and lipoprotein-cholesterol than those encountered in Western populations. A final limitation that is worth mentioning is that we do not have reliable information on the CAD mortality of the five Tanzanian populations.

In conclusion, we have shown that, in the present study population composed of five Tanzanian tribes and one Dutch group, SFA-status is positively related to the TC/HDLC ratio, while the linoleic acid and PUFA-status are negatively related to the TC/HDLC ratio. These findings contrast with the insignificant relation of SFA-intake with TC/HDLC, but are in line with the negative relation of PUFA-intake with TC/HDLC, as established in Western populations. Consequently, the TC/HDLC ratio is a questionable CAD risk marker, because in meta-analyses of randomized controlled trials, partial replacement of dietary SFA for linoleic acid, the dominating dietary PUFA, does not change CAD risk. We contend that there are many lifestyle factors, not SFA-intake alone, determining SFA-status, and that, in the end, it is the interaction with many other lifestyle factors that determines whether SFA-intake and SFA-status have relevant contributing effects on systemic low grade inflammation, changes in lipoprotein metabolism and CAD risk. The present outcome may in reality teach us to consider the health effects of the entire diet together with many non-dietary lifestyle factors, as opposed to the reductionist approach of studying the effects of single nutrients, such as SFA or PUFA.

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CHAPTER 2.3

Comment on the report 'Dietary Fats and Cardiovascular Disease'.

A Presidential Advisory From the American Heart Association (AHA)'

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Abstract

Recently, the American Heart Association (AHA) published a meta-analysis emphasizing their earlier recommendation to limit the intake of saturated fatty acids (SFA). SFA should be replaced with unsaturated fat, especially polyunsaturated fat, to lower the incidence of heart disease. Such replacement is claimed to reduce the risk for cardiovascular events by about 30%; a risk reduction comparable to treatment with statins. The AHA also advises against coconut oil consumption because it increases LDL-cholesterol and ´has no known offsetting favorable effects'. We argue that the LDL-cholesterol concentration is still a soft endpoint, not a disease, while there are no studies showing unfavorable effects of coconut oil on hard endpoints. The AHA extensively motivates the exclusion of studies for their meta-analysis, but does not apply stringent criteria in the choice of the four trials that ultimately constitute the backbone of their meta-analysis. One of these was not a randomized controlled trial, while another suffered from 'performance bias'. The largest negative trial was excluded, amongst others, because it did not last at least two years. The AHA meta-analysis conveys the notion of 'cherry picking'. There are at present at least nine expert reviews that failed to find a clear link between SFA, cardiovascular mortality and total mortality. We argue that individuals with the metabolic syndrome should be careful with dietary SFA and carbohydrates, since they synthesize SFA *de novo* from carbohydrates and spare dietary SFA. The high risk of individuals with the metabolic syndrome is no reason to limit SFA intake of the genuinely healthy population. Some SFA are definitely pro-inflammatory, but a balanced diet also contains anti-inflammatory components.

Keywords

Saturated fatty acids, cardiovascular disease, coconut oil, LDL-cholesterol, polyunsaturated fatty acids, metabolic syndrome, inflammation, CRP.

Declaration of no conflict of interest

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Introduction

The 2015–2020 'Dietary Guidelines for Americans' advice to limit saturated fatty acid (SFA) intake to <10% of daily calories, while the 2013 American Heart Association (AHA)/American College of Cardiology lifestyle guidelines advice to further limit SFA to 5–6% of calories for individuals with elevated LDL-cholesterol (LDL-C) levels [1]. In 2015, the Dutch Health Council abandoned the advice to consume less than 10% of energy as SFA, in favor of the 'replacement of butter, hard margarines, and cooking fats by soft margarines, liquid cooking fats, and vegetable oils' [2].

Recently, the AHA published a meta-analysis [1] emphasizing their earlier recommendation to limit SFA intake. In this 2017 'Presidential Advisory on dietary fats and cardiovascular disease (CVD)', the AHA strongly recommends to lower SFA intake and replace SFA with unsaturated fat, especially polyunsaturated fat (PUFA), to lower the incidence of heart disease. Such replacement is claimed to reduce the risk for cardiovascular events by about 30%; a risk reduction comparable to treatment with statins. In addition, the AHA advises against the use of coconut oil because it increases LDL-C levels and ´has no known offsetting favorable effects' [1]. The simultaneous raising of HDL-cholesterol (HDL-C) [3] is acknowledged but ignored because changes in HDL-C, either by drugs or diet, 'can no longer be directly linked to changes in CVD, and therefore the LDL-C raising effect should be considered on its own' [1].

We are at present confronted with even further deviating opinions regarding SFA and heart disease. For instance, a recent (2017) comment on currently available systematic reviews and meta-analyses of observational studies concluded that there is no association between SFA consumption and all-cause mortality, coronary heart disease (CHD), CHD mortality, ischemic stroke nor type 2 diabetes, in healthy adults [4], while a recent (2017) meta-analysis of randomized controlled trials (RCTs) [5] concluded that replacing SFA with mostly n-6 PUFA is unlikely to reduce CHD events, CHD mortality or total mortality. The latter meta-analysis claims to have excluded inadequately controlled trials and to have exclusively pooled the results of adequately controlled trials. The study found neither a beneficial nor an adverse effect on CHD events, CHD mortality and total mortality.

In this contribution, we comment on the recent AHA advice, discuss the major recent (2017) literature on the SFA-CHD connection and put the consumption of SFA in an evolutionary context.

Is coconut oil unhealthy?

There are, to our knowledge and that of others [6], no studies showing adverse effects of coconut oil on hard endpoints. Vijayakumar et al. [7] conducted a randomized study on the use of 'ordinary' coconut oil versus sunflower oil for cooking. The study population was composed of 200 patients with stable CVD on standard medical care, living in India. The oils were given to the subjects and their family members. After a two-year period, they did not find any differences in anthropometric, biochemical (e.g. total-, HDL-, LDL-cholesterol; triglycerides; and hsCRP) and vascular functions (flow mediated vasodilatation), or cardiovascular events. The AHA advisory does not mention this study, possibly because it did not last more than two years (see below).

We also argue that the LDL-C concentration is still a soft endpoint, not a disease, although many investigators are convinced by the causal involvement of the LDL-C concentration in CVD. However, an LDL-C concentration increase, observed in some RCTs and meta-analyses, might as well be linked with a lower CHD risk (e.g. in RCTs of fish oil and SGLT2 inhibitors), whereas an LDL-C decrease may be linked to a higher CHD risk (e.g. RCTs of hormone replacement therapy) [8]. Statins reduce LDL-C levels and CVD risk but also reduce inflammation, as witnessed from the simultaneous drop of hsCRP. Inflammation changes our metabolism, including the compositions of LDL and HDL. The serum triglyceride concentration increases, HDL-C decreases and both 'small dense' LDL and 'dysfunctional' HDL emerge [8]. The hypothesis of atherosclerosis as being an inflammatory disease dates back to 1859 when R. Virchow noticed that `an inflammation of the inner arterial coat is the starting point of the so-called atheromatous degeneration'. The concept became widely known by a paper of Ross in 1999 [9], that has been cited about 27,000 times). The recent CANTOS trial showed that targeting interleukin-1 beta in patients with previous myocardial infarction, with generally well-controlled LDL-C levels, and hsCRP above 2 mg/L, reduces hsCRP and the rate of recurrent cardiovascular events, without changing LDL- and HDL-cholesterol concentrations [10].

AHA meta-analysis accused of 'cherry picking'

Currently, there are at least nine expert reviews that have failed to demonstrate a clear link between SFA, cardiovascular mortality and total mortality. Short

descriptions of the included trials in these meta-analyses and their conclusions can e.g. be found in references [11], [12] and [13]. Upon comparison, it should, however, be appreciated that the AHA advisory chose cardiovascular events, including myocardial infarction and angina, as endpoint, whereas the more conclusive 'hard' endpoints, including myocardial infarction, stroke and cardiovascular and total mortality, were mostly investigated by other meta-analyses [11].

Selective omission of studies because of 'poor quality', possibly inspired by biased opinion, also named 'cherry picking' [11, 14], seems to have become the new trend and is in the center of the current discussion [4, 11–23]. The AHA advisory excluded most of the existing trials to arrive at a final selection of four and they did not provide clear detailed *a priori* inclusion criteria [11, 14]. Exclusion of currently available studies is extensively explained, but they did not apply equally stringent criteria for choosing the four trials on which their meta-analysis is ultimately based (see below). Detailed *a priori* criteria are a prerequisite for a meta-analysis according to Cochrane guidelines. The following may be cited from the 'Study quality guide' of 'The Cochrane consumers and communication review group': 'A systematic review is only as good as the studies upon which it is built. Including biased studies in a systematic review can therefore produce misleading results. Even if high quality methods are followed for the conduct of the review itself, if studies with serious biases are included and these are not adequately accounted for or acknowledged, poor quality evidence will arise from the review' [24].

More specifically, the AHA advisory notes that they limited the selection to trials that: 1) compared high SFA with high PUFA intake; 2) did not include *trans* unsaturated fat as a major component; 3) controlled the dietary intake of the intervention and control groups; 4) had at least two years of sustained intake of the assigned diets; 5) proved adherence by objective biomarkers such as serum cholesterol or blood or tissue levels of PUFA; and 6) collected and validated information on cardiovascular or coronary disease events. With these criteria, the included studies were: the study of the 'Wadsworth Hospital/Veterans Administration Center in Los Angeles' by Dayton et al. (846 men); the 'Oslo Diet Heart Study' (412 men), the study by the British 'Medical Research Council' (393 men) and the 'Finnish Mental Hospital Study' (1,222 men and women). The total number of subjects was 2,873 and the number of cases was 719 [1].

Given the above criteria and final choices, opponents criticize, amongst other, the inclusion of the poorly controlled 'Finnish Mental Hospital Study', which is not an RCT and has been omitted in all major reviews since 2014. The trial might e.g. have been biased by the use of an antipsychotic drug that was especially taken by the control group and was later found to increase the risk of cardiac death. Because of its large beneficial outcome and weight (31.66%), the inclusion of this study is likely to have driven the conclusions of the AHA advisory to a large extent [11].

On the other hand, the very large (originally 9,570 subjects) 'Minnesota Coronary Experiment' was excluded, among other reasons, because it did not last at least two years, while the outcomes of the 'Dietary Approaches to Stop Hypertension' (DASH; not aimed at fatty acids) study, not lasting for more than five months, were accepted as an example of an 'overall healthful dietary pattern' [11]. In a recent reanalysis of the 'Minnesota Coronary Experiment', replacing SFA with linoleic acid effectively lowered serum cholesterol, but did not support the hypothesis that this reduction translated to a lower risk of death from CHD or all causes. More precisely, it was found that, in participants older than 65 years, a 30 mg/dL (0.79 mmol/L) decrease in serum cholesterol was associated with a 35% higher risk of death (HR 1.35, 95% CI 1.18–1.54), whereas among people aged under 65 at baseline, there was no such relation (1.01, 0.88–1.16) [25].

The inclusion of the 'Oslo Diet Heart Study' into the AHA advisory is also criticized [14]. This study holds 27.95% of the weight in the AHA meta-analysis, thus amounting 59.61% of the total weight when taken together with the afore-mentioned 'Finnish Mental Hospital Study'. The 'Oslo Diet Heart Study', though randomized, suffered from a 'performance bias' [26], with the intervention group receiving 'continuous instruction and supervision', while the control group received no counseling at all. This would be the equivalent of a drug trial without placebo control, and, moreover, unblinded, since the physicians who referred the studied patients to the main investigator were obviously aware of their assignment to either intervention or control. A resulting bias is plausible, since the intervention group reported a very low sugar intake [14].

Other comments regarding the four selected trials comprise the AHA endpoint of only CHD events. The absence of an analysis of total mortality raises questions about the occurrence of adverse effects [14].

Recent (2017) studies on dietary SFA and cardiovascular disease

The AHA advisory did not change our opinion [27–29] regarding the influence of dietary SFA on CHD. We still argue against the causality of the relation between SFA intake, cholesterol and CHD mortality. Our concern is the projection of this line of reasoning on the general, low-risk, healthy population [28,30]. Individuals with the metabolic syndrome (insulin resistance syndrome) are known to *de novo* synthesize fat, notably palmitate, from polar precursors, especially glucose. In this condition, also referred to as pre-diabetes, the sparing of dietary fat and its *de novo* synthesis are among the various factors in the initiation of an inflammatory program, as recently extensively reviewed by Reilly and Saltiel [31]. As discussed elsewhere [27–29] dietary- and *de novo* synthesized-palmitate interact with toll like receptor-4 to initiate an inflammatory cascade by as yet incompletely delineated mechanisms.

A recent paper by Chiu et al. [8] strengthens the above notion that especially individuals with 'LDL phenotype B', a feature of the metabolic syndrome, should limit both their SFA- and carbohydrate-intakes, since dietary SFA and carbohydrates are likely to interact. Individuals with the LDL phenotype B present high levels of small-dense LDL particles. The latter, and also medium sized LDL particles, are components of 'atherogenic' dyslipidemia, and associate with CVD outcomes more strongly than larger LDL particles. It was concluded that 'saturated fat may have heterogeneous effects on levels of atherogenic LDL particles that may depend on the amount of saturated fat consumed, the dietary context, particularly concomitant carbohydrate intake, and/or predisposition to atherogenic dyslipidemia'. We conclude that there is no convincing evidence that the current recommendations for SFA should apply to healthy subjects with LDL phenotype A with predominance of large, buoyant LDL particles, who do not suffer from the metabolic syndrome and are at low CHD risk.

Other recent studies published in the Lancet [32–34], seem to have debunked the 'lipid hypothesis' that started I 1958 originating from the Seven Countries Study of Ancel Keys. These Lancet papers on the 'Prospective Urban Rural Epidemiology' (PURE) study did, however, meet serious criticism (see below). The PURE study is a very large, epidemiological cohort study of individuals aged 35–70 years in 18 countries in five continents. The study includes high-income, medium-income and low-income nations. Enrolment was from 2003–2013 and the median follow-up was 7.4 years. The authors associated dietary intakes of 135,335 individuals with cardiovascular mortality, disease and events and non-CVD mortality. A very high carbohydrate intake (>70 energy%) was associated with a higher risk of total mortality, but not with CVD risk, whereas total fat, SFA, MUFA and PUFA were related to a lower total mortality. Total fat, SFA, MUFA and PUFA were not associated with CVD, myocardial infarction nor cardiovascular disease mortality, whereas SFA exhibited an inverse association with stroke [32, 34]. In addition, the PURE study investigated the association between nutrients and CVD risk markers in 125,287 participants [33, 34]. The outcome confirmed that SFA intake is associated with higher LDL-C but also with higher HDL-C and lower triglycerides, total cholesterol/HDL-C ratio, triglycerides/HDL-C ratio and ApoB/ApoA1 ratio. Modelling studies showed that replacing SFA for unsaturated fats might improve some risk markers (total cholesterol, LDL-C, triglycerides/HDL-C ratio and blood pressure) but may also worsen others (HDL-C and triglycerides). It was concluded that 'focusing on a single lipid marker such as LDL-C alone does not capture the net clinical effects of nutrients on cardiovascular risk. Based on the lipid profile, a reduction of SFA intake below 10 energy% was not supported [33, 34].

It must be noted that, based on its design, lack of causality and data analysis; the PURE study has been seriously criticized [35, 36]. Some of the findings, e.g. the LDL-C raising effect of PUFA, are at odds with well-controlled feeding trials [36]. The strength of the PURE study, comparing dietary data with disease outcomes across a broad range of countries and geographical regions, constitutes at the same time its weakness, since such a design with many variables profoundly increases the chance of residual confounding from parameters like wealth, socioeconomic status and access to health care [35, 36]. For instance, the relation of SFA intake with lower total mortality might as well reflect an association between higher consumption of animal fat (i.e. wealth) and access to either a hospital or medical care [35].

Evolutionary approach

From an evolutionary point view, there is also no reason to reduce the consumption of a nutrient that we have always eaten, in favor of the consumption of an amount of linoleic acid (advice 5–10 energy% [37])

that we have never eaten in our whole evolutionary past [38, 39]. There should be good scientific evidence for such a recommendation, which is currently not the case. The 'precautionary principle' to risk management is applicable here. This principle states that if an action or policy has a suspected risk of causing any harm, either to the public or to the environment, in the absence of scientific consensus, the burden of proof that it is not harmful falls on those taking that action [40].

An alternative advice to the *healthy population* might be to take a statin, aspirin, antihypertensive, or even an SGLT2 inhibitor, to lower CVD and other risks. There is basically no difference in the required level of evidence for such drugs in primary prevention, compared with a dietary advice that is remote from what we are based on from an evolutionary point of view. The 'unnatural´ advice to lower SFA in favor of linoleic acid should be regarded as 'treatment of the healthy population'. It may be argued that such options, i.e. drugs or unnatural diet, are nowadays needed for prevention because of our deviation from a healthy lifestyle, which is more than diet alone [30]. We contend that lifestyle changes, not the masking of an unhealthy lifestyle with unnatural diets or drugs, are definitively better choices, both from the point of view of the individual and the society as a whole.

New studies?

What does it take to settle the differences? It is unlikely that new, impeccable, studies will be conducted for many reasons, including high costs, 20,000–30,000 participants needed, feasibility to deliver diets to such large numbers, adherence to intervention for at least five years, declining CVD incidence rates because of improved lifestyle, and better medical treatment [1, 14]. The current intervention studies are what they are and, for all practical purposes, one has to reach a(n) (interim) conclusion.

Conclusions

Individuals with the metabolic syndrome should be advised to limit dietary SFA intake, since they synthetize SFA *de novo* from carbohydrates and spare dietary SFA [27–29]. The high risk of individuals with the metabolic syndrome is no reason to limit SFA intake by the genuinely healthy population. Some SFA, for example palmitate, are definitely pro-inflammatory, but a balanced diet also contains anti-inflammatory components, as extensively outlined elsewhere [27–30, 41].

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CHAPTER 3.1

Kinetics of plasma- and erythrocyte-astaxanthin in healthy subjects following a single and maintenance oral dose

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Abstract

Aim & background: Astaxanthin is a unique carotenoid of predominantly marine origin providing the pink-red color to certain microalgae and accumulating in various animals higher in the food chain. It is an antioxidant without pro-oxidant properties or known side-effects following oral intake.

Methods: We investigated astaxanthin kinetics in plasma and erythrocytes (RBC) of four healthy adults after a single oral 40 mg dose. Plasma- and RBC-astaxanthin were measured during 72 h. Subsequently, an 8 mg/day dose was given during 17 days. Plasma- and RBC-astaxanthin were measured each morning.

Results: Plasma-astaxanthin reached a peak (from 79 to 315 nmol/L) after 8 h and then declined (half-life, 18 h). Within 72 h, plasma-astaxanthin had returned to baseline. RBC-astaxanthin reached a peak (from 63 to 137 nmol/L packed cells) at 12 h and subsequently disappeared (half-life, 28 h). During the daily dose, plasma-astaxanthin increased until day 10 (187 nmol/L) and then decreased to a steady concentration similar to that reached after 2 days. RBC-astaxanthin appeared to be highly variable (group median concentration, 86 nmol/L packed cells).

Conclusion: We found high intra- and inter-individual variations, especially in RBC, possibly due to non-standardized time difference between astaxanthin intake and sampling, fluctuating background intake from the diet, variable bioavailability, large distribution volume, degradation or others. Oral astaxanthin is rapidly absorbed and incorporated into RBC. The subsequent rapid decline suggests that, for a higher-than-baseline status, astaxanthin should be taken daily, at least in an early phase when total body equilibrium, if any, has not been reached yet.

Keywords
Antioxidant; carotenoid; humans; half-life; status; absorption.

Abbreviations
RBC, red blood cells; CVD, cardiovascular disease.

Introduction

A diet rich in natural antioxidants supports health [1, 2] strengthens the antioxidant network and is thereby associated with lower oxidative stress and inflammation, leading to decreased risk of cardiovascular disease (CVD), neurodegenerative diseases, certain cancers and other diseases [3]. Astaxanthin has recently received attention for its potent antioxidant activity [4] without pro-oxidant properties [5]. It is a unique carotenoid belonging to the xanthophyll family, synthesized by plants and algae providing them with a pink-red color [6] and accumulating in certain animals higher in the food chain, such as flamingoes, salmon, shrimps and crayfish [7]. Natural astaxanthin is optically distinct from synthetic astaxanthin. It is commercially available as a food supplement from the algae *Haematococcus pluvialis* [8].

Orally administered astaxanthin incorporates into both plasma and erythrocytes (RBC) of healthy subjects, improves RBC antioxidant status and decreases membrane phospholipid peroxidation after a 12-week daily supplementation [9]. Following its ingestion, astaxanthin has been shown to reach a peak in plasma at about 7 h and to decline with a median half-life of about 21 h [10]. The kinetics of astaxanthin in RBC are currently unknown. We investigated the kinetics of astaxanthin in both plasma and RBC after a single oral 40 mg dose and its distribution in both compartments during a 17-day 8 mg/day maintenance dose.

Materials and methods

Study design and study group

Four apparently healthy volunteers (1 male, 3 females) aged 29–41 years (weight 59–70 kg, height 1.73–1.80 m, individual body-mass indices, 19.7, 20.7, 22.1 and 22.9 kg/m^2), participated in this pilot intervention study. Throughout the study period, subjects were instructed to maintain their usual lifestyle. Age, weight and length were self reported. All participants received verbal and written explanation of the objectives and procedure of the study and subsequently provided us with written informed consent.

Astaxanthin supplementation

The pilot study consisted of two well-defined parts. In the first part, a single dose of 40 mg astaxanthin (10 soft gel gelatin capsules containing an astaxanthin extract from the algae *Haematococcus pluvialis*; Cyanotech, Hawaii) was given to the 4 subjects together with a fat-containing breakfast, as astaxanthin absorption is improved in the presence of lipid based formulations [11]) Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (as d-alpha tocopherol), 64 µg β-carotene, 40 µg lutein and 72 µg canthaxanthin. The capsules contained glycerol and safflower oil as wetting and filling agents, respectively. Five days after the 40 mg astaxanthin intake, the second part of the pilot was initiated, where the four participants were instructed to take a daily dose of 8 mg astaxanthin during 17 days. The astaxanthin capsules were taken in the evening together with, or just after, a fat-containing meal. Compliance was verbally checked by one of us on the following day.

Sample collection and analyses

EDTA-anticoagulated blood (4 mL) was collected in the morning by venipuncture at baseline and at 2, 4, 6, 8, 10, 12, 24, 32, 48 and 72 h after the single oral 40 mg astaxanthin dose and every morning for 13 days (days 1–5, 8–12 and 15–17) following the daily 8 mg maintenance dose.

EDTA-blood was centrifuged for 10 min at 1,000 g in a cooled centrifuge (4°C) for the separation of plasma and RBC. The EDTA-plasma (200 µL) was transferred to a teflon-sealable Sovirel tube containing 2.75 mL of an antioxidant solution containing EDTA, ascorbic acid, pyrogallol and butylated hydroxytoluene in methanol/water for the preservation of astaxanthin. Following the removal of plasma and buffy coat, RBC were washed three times with 0.9% saline. Phosphate buffered saline (pH=7.4) was subsequently added to prepare an about 50% hematocrit suspension, from which 500 µL were transferred to a teflon-sealable Sovirel tube containing 2.75 mL of the aforementioned antioxidant solution. The remainder was used for a total cell count including a hematocrit measurement (Sysmex, Etten-Leur, The Netherlands).

All tubes were frozen at –20°C until analyses. Plasma- and RBC-astaxanthin were determined with HPLC/VIS using previously described procedures [12, 13]. Briefly, this method includes hexane extraction, evaporation to dryness under nitrogen, and re-dissolution in methyl tert-butyl ether (MTBE) and ethanol. From this mixture, 50 µL are injected into the HPLC. The analytical system was composed of a Carotenoid 250 × 2.1 mm ID column (YMC, Japan) with HPLC/VIS detection operated at a flow rate of 0.3 ml/min, using a gradient of solvent A (methanol: MTBE: H_2O =

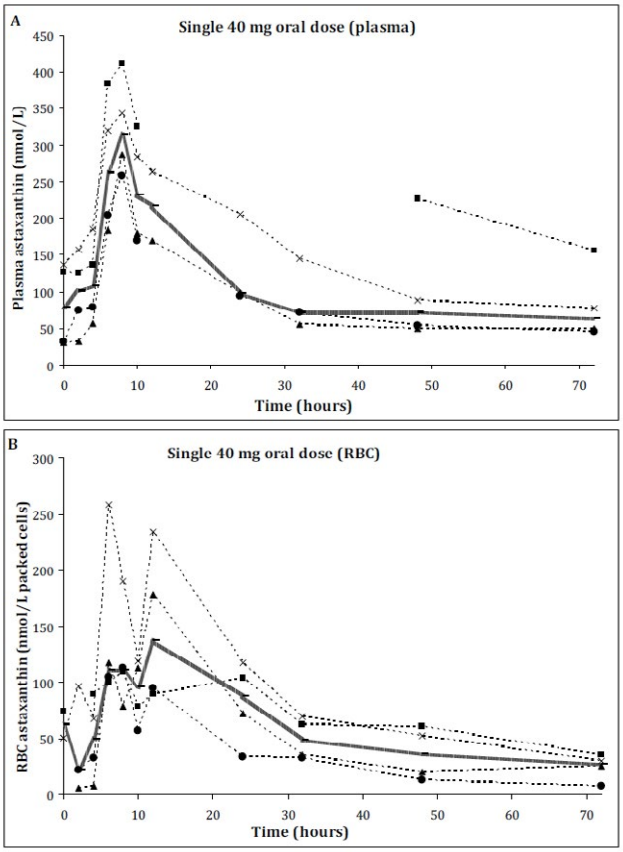

Figure 1. Courses of astaxanthin in plasma (A) and erythrocytes (B) after a single 40 mg astaxanthin oral dose.
Healthy subjects (n=4) took a single 40 mg astaxanthin oral dose (from Haematococcus pluvialis; Cyanotech, Hawaii). Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (as d-alpha tocopherol), 64 µg β-carotene, 40 µg lutein and 72 µg canthaxanthin. Dotted lines represent data of the 4 individuals and the bold line represents their medians. Abbreviation: RBC, red blood cells.

81:15:4 v/v/v) and solvent B (methanol: MTBE = 7:93 v/v) at a detection wavelength of 450 nm.

The RBC-astaxanthin content (in nmol/L packed cells) was calculated by dividing the measured RBC-astaxanthin concentration by the hematocrit of the washed RBC suspension.

Results

The between-series plasma-astaxanthin CVs (n=6) at mean levels of 42 and 111 nmol/L amounted to 5.3 and 3.8%, respectively, implying that the analytical reference change values (2.8 times the SDs of 2.2 and 4.2 nmol/L, respectively) [14] are about 6 and 12 nmol/L, at the low and higher levels, respectively.

Plasma- and RBC- astaxanthin kinetics after a single 40 mg oral dose

The individual and median courses of plasma- and RBC-astaxanthin up to 72 h after the single 40 mg dose are presented in Figure 1. Large intra- and inter-individual variations in the courses were observed for plasma- and notably for RBC-astaxanthin, (panels A and B). Because of the variations in time to reach the peak values and also peak heights, and in view of the small sample size, we refrained from statistical analyses. Nevertheless, all study subjects responded well beyond the analytical variation, as their differences between baseline and peak levels were well beyond the reference change value for analytical variation.

In all four subjects, plasma-astaxanthin (panel A) reached a peak (from a baseline median of 79 to 315 nmol/L) after 8 h and subsequently declined with an estimated half-life of 18 h. Within 72 h, plasma-astaxanthin had returned to baseline (median, 64 nmol/L). This is in agreement with Østerlie et al., who reported a plasma-astaxanthin peak at about 7 h following a 100 mg astaxanthin oral dose and a half-life of about 21 h [10]. RBC-astaxanthin (panel B) reached a peak (from 63 at baseline to 137 nmol/L packed cells) at 12 h and subsequently

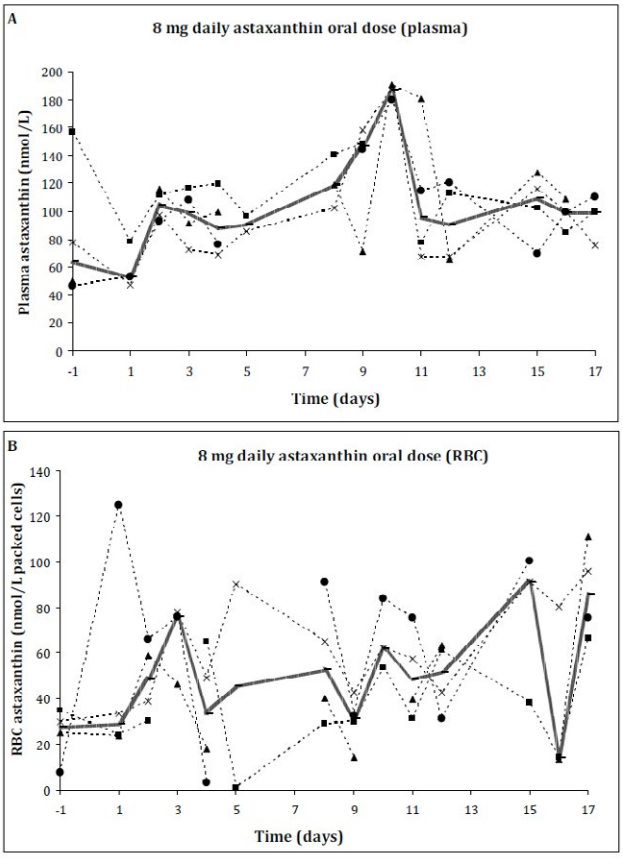

Figure 2. Courses of astaxanthin in plasma (A) and erythrocytes (B) during a 17-day maintenance dose of 8 mg astaxanthin/day.

Healthy subjects (n=4) took a daily dose of 8 mg astaxanthin (from Haematococcus pluvialis; Cyanotech, Hawaii) during 17 days. Supplementation started 5 days after the single 40 mg oral dose. Baseline levels (day -1) were taken from the astaxanthin concentrations at 72 hours after the single 40 mg astaxanthin oral dose (see Figure 1). Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (as d-alpha tocopherol), 64 µg β-carotene, 40 µg lutein and 72 µg canthaxanthin. Dotted lines represent data of the 4 individuals and the bold line represents their medians. Abbreviation: RBC, red blood cells.

disappeared with a half-life of about 28 h. Individual summits were reached after 6, 8, 8, and 12 h. Also for RBC, the baseline was reached within 72 h (median, 27 nmol/L packed cells). The median percentage astaxanthin found in RBC throughout the 72 h observation period (with the plasma concentration set at 100%) amounted to 44% (range of individual medians: 26–56%). These RBC-plasma ratios, subject to a large variation, are comparable to the median of 43% (range 35–48%) that we estimated from the data from Nakagawa et al. [9] who supplemented healthy adults with 6 and 12 mg oral astaxanthin/day for 12 weeks.

Plasma- and RBC-astaxanthin during a 17-day 8 mg/day oral maintenance dose

The individual and median courses of plasma- and RBC-astaxanthin during the 17-day maintenance dose are presented in Figure 2. Supplementation started 5 days after the single 40 mg oral dose. Baseline levels were taken from astaxanthin concentrations at 72 h after the single 40 mg astaxanthin oral dose. Plasma-astaxanthin (panel A) increased slowly until day 10, reaching a maximum of 187 nmol/L. After this peak, plasma astaxanthin decreased to reach a steady concentration similar to that reached after 2 days. RBC-astaxanthin (panel B) appeared to be highly variable, giving rise to a group median concentration of 86 nmol/L packed cells throughout the 17 days supplementation period. The median percentage astaxanthin found in RBC throughout the 17 days maintenance dose (with the plasma concentration set at 100%) was 49% (range of individual medians: 28–71 %). This value is in agreement with the median of 44% (range of individual medians: 26–56%) of the study group throughout the 72 h observation period following the single 40 mg dose (see above), and comparable to the median of 43% (range 35–48%) that we estimated from the data from Nakagawa et al. [9]. Large intra- and inter-individual variations

were observed for plasma- and RBC-astaxanthin, notably for the latter, during the 17 days supplementation period. This may be partly due to the low dose compared with the background intakes (from food). For instance, the median inter-individual plasma-astaxanthin CV during the 17 days maintenance dose amounted to 20% (range: 3–46%), and for RBC-astaxanthin this CV was 40% (range: 22–212%).

Discussion and conclusions

The short plasma- and RBC-astaxanthin half-lives of 18 and 28 h, respectively, suggest the necessity to take astaxanthin on a daily basis to maintain a higher-than-baseline steady state, at least in the initial phase of supplementation. This early phase might in part be influenced by the tendency of astaxanthin to incorporate into all bodily cell membranes. Astaxanthin is likely to be subject to a large distribution volume, in which probably not all compartments are reached at equal rates. Both the single and maintenance oral doses gave rise to large intra- and inter-individual biological variations, especially in the RBC compartment. The variability in responses may e.g. derive from non-standardized time difference between astaxanthin intake and blood sampling, a fluctuating background intake from the diet, variable bioavailability, large distribution volume, (induced) degradation and others. High intra- and inter-individual bioavailability of carotenoids has been previously reported [15–17] and the present data on astaxanthin appear as no exception to this rule.

Orally administered astaxanthin is rapidly absorbed and becomes rapidly incorporated into RBC. The subsequent rapid decline suggests that, for a higher-than-baseline status, astaxanthin should be taken daily, at least in an early phase when total body equilibrium, if any, has not been reached yet.

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CHAPTER 3.2

Supplementation of patients with sickle cell disease with astaxanthin increases plasma- and erythrocyte-astaxanthin and may improve the hemolytic component of the disease

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Abstract

Introduction: Sickle cell disease (SCD) is characterized by hemolytic and vaso-occlusive components. Astaxanthin is a carotenoid of marine origin, without pro-oxidant properties.

Methods: In this open label pilot study, we investigated whether orally administered astaxanthin incorporates into erythrocytes (RBC) of SCD patients and studied the effect on hematological and clinical chemical parameters. Ten SCD patients (6–52 years) in Sint Maarten received 8–12 mg astaxanthin during 3 months.

Results: Baseline plasma- (33 nmol/L) and RBC- (11 nmol/L packed RBC) astaxanthin increased to 225, 174, 167 nmol/L (plasma) and 149, 100, 71 nmol/L packed RBC (RBC) at 1–3 months, respectively. Reticulocytes decreased from baseline and 2 months (9.5 and 8.8%) to 3 months (5.6%), MCV from 2 to 3 months (88 to 86 fL), MCH from baseline to 3 months (30 to 28 pg) and RDW from baseline and 2 months (19.2 and 19.0%) to 3 months (16.7%). Plasma arginine decreased from 2 to 3 months (46.6 to 39.4 µmol/L). Asymmetric dimethylarginine did not change. Reticulocytes at baseline correlated with relative changes in reticulocytes from baseline to 3 months. Relative changes in reticulocytes correlated with relative changes in RBC, RDW, LDH, ALAT, but not hematocrit, within the same period.

Conclusions: Astaxanthin incorporates into SCD RBC and may favorably affect the hemolytic component. A larger randomized controlled trial is indicated, using similar or higher dose, preferably during more than 3 months, concomitant with (other) low dose antioxidants (vitamin E, beta-carotene, vitamin C, folic acid), minerals (zinc, if necessary, selenium), arginine, fish oil and vitamin D.

Keywords

Antioxidant; hemolytic component; reticulocytes; arginine; asymmetric dimethylarginine

Abbreviations

ADMA, asymmetric dimethylarginine; ALAT, alanine aminotransferase; HbS, sickle-cell hemoglobin; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; NO, nitric oxide; RBC, red blood cell; RDW, RBC distribution width; ROS, reactive oxygen species; SCD, sickle cell disease.

Introduction

Sickle cell disease (SCD) is a heterogeneous disorder that is mechanistically characterized by hemolytic and vaso-occlusive components. The latter gives rise to cumulative ischemic organ damage [1] that may occasionally precipitate to painful vaso-occlusive crises; all jointly contributing to diminished quality of life and early death [2]. The hemolytic component may find an important trigger in the generation of reactive oxygen species (ROS) by HbS close to the lipid peroxidation-sensitive erythrocyte (RBC) membrane [3, 4], ending up in hemolysis [3]. The vaso-occlusive component may be largely driven by the aforementioned hemolytic component. The resulting enhanced sickle RBC turnover in HbSS patients (RBC half-life 5–10 days [2]) gives rise to a very young RBC population with a tendency to adhere to activated vascular endothelium [5], notably in the post-capillary venules [6]. While sickle RBCs activate the vascular endothelium, the activated endothelium expresses adhesion molecules, providing a pro-adhesive surface for young RBCs and leukocytes [7]. The hemolytic component also negatively affects the hemodynamic stability by reducing nitric oxide (NO) availability [8] through different mechanisms, including NO scavenging by cell-free hemoglobin [9], increased circulating arginase activity [10, 11], low levels of circulating arginine (NO precursor) [12] and inhibition of NO-synthase through the presence of increased plasma asymmetric dimethylarginine (ADMA) [13, 14]. The elevated plasma ADMA levels in SCD patients relate to the hemolytic component [13, 15] and may derive from proteolysis following hemolytic stress [16, 17].

Targeting the hemolytic component, and notably oxidative stress, by amelioration of the devastating vaso-occlusive component seems a logical intervention strategy for SCD. Oxidative stress may indeed aggravate the symptoms of SCD [18] and may be counteracted by naturally occurring low-toxicity nutrients [19, 20]. Probably due to the increased and constant need to neutralize the oxidative stress, SCD patients exhibit important depletions of various antioxidants [21], including retinol, alpha-tocopherol, and β-carotene, together with a reduced activity of RBC Cu/Zn-superoxide dismutase and Se-glutathione peroxidase [22]. Various trials with naturally occurring antioxidants with promising outcomes have been reported, including those with vitamin E [23], curcuminoids [24], aged garlic extract [25], N-acetylcysteine [26] and zinc [27].

Astaxanthin is a unique carotenoid. The natural form, predominantly of marine origin, is an antioxidant without pro-oxidant properties [28–30] or side-effects after oral intake [31]. It belongs to the xanthophyll family, providing the pink-red color to certain microalgae (i.e. *Haematococcus pluvialis*) [32] and accumulates in various animals higher in the food chain such as flamingoes, salmon, shrimps and crayfish [33]. The astaxanthin molecule spans the phospholipid double layer of cell membranes due to its two polar head groups that are interspaced by a branched carbon atom chain containing 9 conjugated double bonds. Among the carotenoids that have been shown to incorporate into RBCs of healthy subjects, we can find β-carotene [34, 35], lutein [36] and astaxanthin [37]. Astaxanthin has been found to enhance the immune response [38, 39], to decrease oxidative damage-related symptoms [37, 40] and has been proven effective in several diseases and conditions, such as Alzheimer's disease [41], obesity [39], asthma, enlarged prostate [42], osteoarthritis and rheumatoid arthritis [43].

Due to its unique antioxidant properties, we hypothesized that astaxanthin supplementation might ameliorate the hemolytic component of SCD. For this purpose, we performed an open-label pilot study with 10 SCD patients in Dutch Sint Maarten (Caribbean Sea; 18.0237°N 63.0458°W). The transatlantic slave trade introduced the sickle gene into the former Dutch Caribbean, giving rise to an estimated heterozygote (HbAS) prevalence of 6.84% in Sint Maarten, 2.65% in Aruba, and 5.03% in Curaçao. It was estimated that Dutch Sint Maarten harbors 122 SCD patients (40 HbSS and 82 HbSC) among its 50,300 inhabitants [44]. We investigated the effect of a daily 8–12 mg oral dose during 3 months, on plasma- and RBC-astaxanthin levels (primary goal) and several hematological and clinical chemical parameters (secondary goal), including reticulocyte count, mean corpuscular volume (MCV), RBC distribution width (RDW), lactate dehydrogenase (LDH) and ADMA.

Materials and methods

Study design and study group

In this open label pilot intervention study, we included 10 laboratory-confirmed SCD outpatients (7 adults, 3 children, of which 3 males and 7 females, mean age 31 years, range 6–52 years) from the Sint Maarten Medical Centre, with a mean height of 170 cm (range 155–195 cm), mean weight of 58 kg

(range 29–91 kg) and mean body mass index of 21 kg/m^2 (range 16–27 kg/m^2). Exclusion criteria were painful crisis and blood transfusion in the preceding 4 weeks and 4 months respectively, pregnancy or the desire to get pregnant in the following 3 months, lactation, active infections and/or auto-immune inflammatory diseases. Drop-out criterion was blood transfusion during the study. Most of the information was obtained from their medical records. Weight and length were measured on the spot.

All participants received verbal and written explanation of the objectives and procedure of the study and subsequently provided us with written informed consent for being included in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. All procedures involving human subjects were approved by the Sint Maarten Medical Center-Medical Staff Medical Ethical Committee (1A-09-2011, dated September 6, 2011).

Astaxanthin supplementation

Patients were instructed to take a daily dose of 8–12 mg astaxanthin (soft gel gelatin capsules containing an astaxanthin extract from the alga *Haematococcus pluvialis*; Cyanotech) during 3 months. The dose was based on 12 mg astaxanthin/70 kg. Children weighing less than 40 kg took 8 mg astaxanthin/day and adults and children above 40 kg took 12 mg astaxanthin/day. Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (as d-alpha-tocopherol), 64 µg β-carotene, 40 µg lutein and 72 µg canthaxanthin. The capsules contained glycerol and safflower oil as wetting and filling agents, respectively. The capsules were taken in the morning together with or just after a fat-containing breakfast, as astaxanthin absorption is improved in the presence of lipid based formulations [45]. A compliance intake form was handed to all patients to check daily capsules intake.

Sample collection and analyses

Blood (3 mL) and EDTA-anticoagulated blood (4 mL) were collected by venipuncture from fasting subjects at baseline and after 1, 2 and 3 months astaxanthin supplementation. To avoid major variations in the plasma- and/or RBC-astaxanthin levels, patients were asked to visit the hospital for blood sampling in the morning, 20–24 h after the last astaxanthin intake.

Serum was separated by centrifuging the blood for 10 min at 1,200 g. Measurements of CRP, LDH, bilirubin, creatinine and alanine aminotransferase (ALAT) were performed in Sint Maarten Medical Center (Vitros® 5600 Integrated System, Johnson & Johnson, Puerto Rico).

EDTA-anticoagulated whole blood was used for a complete blood cell count (RBCs, white blood cells and platelets) and the measurements of hemoglobin, hematocrit and reticulocytes in the Sint Maarten Medical Center (Cell-Dyn® 3200, Oduber Agencies (Abbott Diagnostics), Curaçao). The remaining EDTA-blood was centrifuged for 10 min at 1,000 g for the separation of plasma and RBCs in a cooled centrifuge (4°C). 200 µL of EDTA-plasma were transferred into a teflon-sealable Sovirel tube containing 2.75 mL of an antioxidant solution (containing EDTA, ascorbic acid, pyrogallol and butylated hydroxytoluene in methanol/water) for the preservation of carotenoids. The remaining EDTA-plasma was subsequently divided in equal portions of about 250 µL and pipetted into 2.5 mL round plastic tubes.

After both plasma and buffy coat were removed, RBCs were washed three times with 0.9% NaCl to prepare packed cells. After washing, the cell mass was suspended in 1 mL phosphate-buffered saline (pH=7.4). From this suspension, 500 µL were transferred into a teflon-sealable Sovirel tube containing 2.75 mL of an antioxidant mix for the preservation of carotenoids. Total cell counts of the washed RBC suspensions were also performed.

All tubes were frozen at –20 °C until transport and analyses in The Netherlands. Transport to The Netherlands was done in dry ice. Plasma- and RBC-astaxanthin were determined with HPLC/VIS in the University Medical Center Groningen (UMCG; The Netherlands), using previously described procedures [46, 47]. For the calculation of RBC-astaxanthin, we corrected the astaxanthin concentration for the hematocrit to obtain the concentration per packed cells.

Plasma arginine, homoarginine, ADMA and symmetric dimethylarginine (SDMA) were determined with reversed-phase HPLC with fluorescence detection in the VU Medical Center Amsterdam (VUmc; The Netherlands) using previously described procedures [48, 49].

Statistics

Statistical analyses were performed with PASW version 18.0 (SPSS Inc, Chicago, IL). Data were analyzed

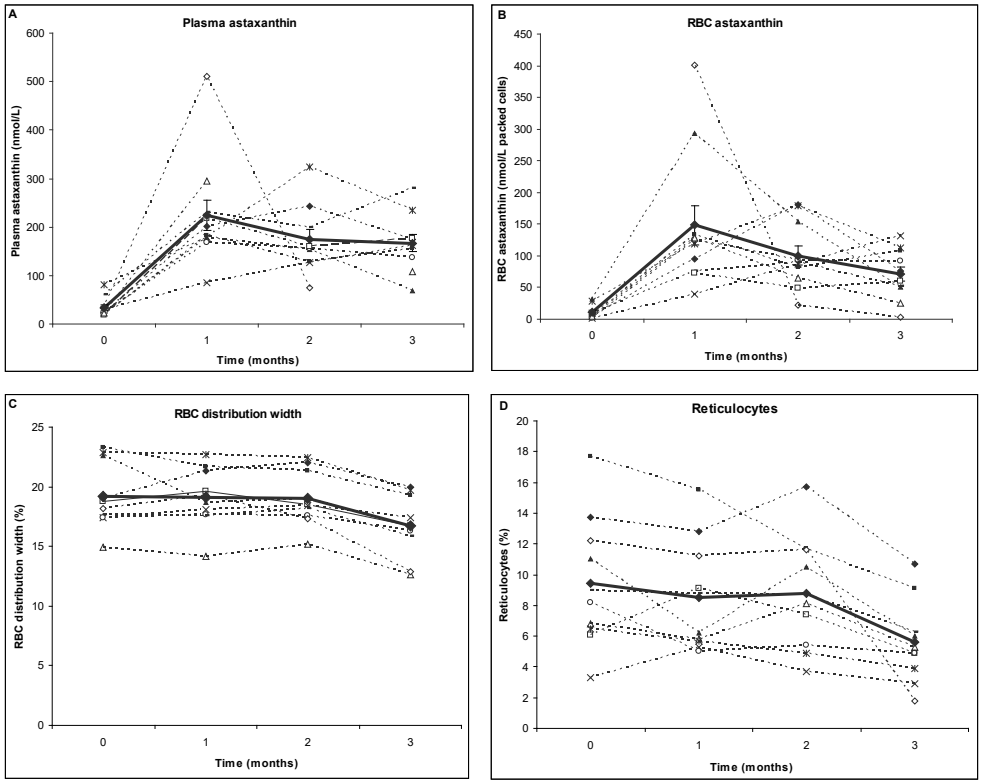

Figure 1. Courses of plasma astaxanthin (panel A) and RBC astaxanthin (panel B), red cell distribution width (panel C) and reticulocytes (panel D) of SCD patients during 3 months oral supplementation with astaxanthin

Patients (n=10) received 8 or 12 mg astaxanthin (12 mg/70 kg) daily during 3 months. Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (d-alpha tocopherol), 64 µg β-carotene, 40 µg lutein and 72 µg canthaxanthin. Dotted lines represent the courses of individual SCD patients; bold lines represent their means. Both plasma- (panel A) and RBC- (panel B) astaxanthin increased from baseline to 1-3 months supplementation. There were high intra- and inter-individual variations in both plasma- and RBC-astaxanthin concentrations. RDW (panel C) decreased from both baseline and 2 months to 3 months, and reticulocytes (panel D) decreased from 2 to 3 months. Abbreviations: RBC, red blood cells, RDW, red blood cell distribution width.

with paired-samples t-tests applying Bonferroni correction for multiple time points to determine whether there were significant longitudinal changes in each of the parameters. Bivariate correlations using Spearman's correlation coefficient were performed between reticulocytes (%) at baseline and the relative change in reticulocytes (%) between 3 months and baseline, and also between the relative change in reticulocytes (%) between 3 months and baseline and the changes in the same period of the other parameters. Linear regression was used to model the relationship between the aforementioned variables, for the calculation of both the slope and intercept of the linear equation from the observed correlations.

Results

All patients had been diagnosed with homozygous SCD (HbSS) in Sint Maarten. The occasionally low MCV suggests that at least 7 of them might have concomitant alpha-thalassemia or (less probably) HbSβ°. Their hemoglobin profiles, as established with HPLC at baseline, did not reveal any HbA. We found that the supplement was well tolerated. There were no signs or complaints of side effects and many of the patients reported spontaneously that they 'felt much better' during the study.

Table 1 Astaxanthin in plasma and erythrocytes together with hematological and clinical parameters during 3 months oral supplementation of sickle cell patients with astaxanthin

		Sampling point			
	Reference values	**Baseline**	**1 month**	**2 months**	**3 months**
Plasma astaxanthin (nmol/L)	–	33.3 (18.5–80.3) [b, c, d]	224.6 (84.3–510.2) [a]	173.9 (75.1–324.3) [a]	166.6 (68.8–281.1) [a]
RBC astaxanthin (nmol/L packed cells)	–	11.2 (1.2–32.1) [b, c, d]	148.5 (39.5–400.9) [a]	100.3 (22.3–180.4) [a]	71.3 (3.4–131.5) [a]
% astaxanthin in RBC (with plasma astaxanthin=100%)	–	36.2 (4.0–149.9) [b]	66.2 (33.2–164.9) [a]	58.7 (29.6–100.1)	51.0 (18.9–80.8)
Hemoglobin (g/dL)	12.0–15.0	8.3 (6.9–10.1)	8.4 (7.0–10.2)	8.7 (8.3–9.7)	8.6 (7.3–11.2)
Hematocrit (%)	37.0–52.0	28.2 (23.6–36.9)	26.1 (21.4–30.3)	27.6 (25.4–30.4)	25.1 (19.3–31.0)
RBC (103/µL)	4.20–6.10	2.85 (2.13–4.16)	3.00 (2.13–4.54)	3.10 (2.30–4.43)	3.20 (2.09–4.86)
Reticulocytes (%)	0.5–2.5	9.5 (3.3–17.7) [d]	8.5 (5.0–15.5)	8.8 (3.7–15.7) [d]	5.6 (1.8–10.7) [a, c]
RDW (%)	11.5–14.5	19.2 (14.9–23.3) [d]	19.1 (14.2–22.7)	19.0 (15.2–22.4) [d]	16.7 (12.0–20.0) [a, c]
MCV (fL)	80.0–99.0	88.4 (74.9–102.0)	85.8 (72.1–103.0)	87.8 (68.6–106.0) [d]	85.5 (69.1–99.6) [c]
MCH (pg)	27.0–31.0	29.8 (24.0–35.3) [d]	28.9 (22.5–36.3)	28.9 (21.4–36.4)	28.0 (20.2–35.5) [a]
MCHC (g/dL)	33.0–37.0	33.7 (30.8–35.7)	33.6 (31.2–37.8)	32.8 (30.3–36.9)	32.5 (29.3–35.6)
WBC (103/µL)	4.8–10.8	15.2 (4.6–50.6)	10.0 (4.4–20.0)	8.9 (3.6–12.2)	9.4 (3.3–14.7)
Neutrophils (%)	37.0–80.0	41.0 (17.2–60.9)	42.2 (27.6–59.7)	45.3 (29.5–59.0)	44.5 (24.9–65.5)
Lymphocytes (%)	10.0–50.0	43.9 (20.6–75.0)	40.3 (26.4–55.2)	37.0 (24.8–47.5)	40.3 (25.6–63.2)
Monocytes (%)	0.0–12.0	8.6 (2.3–13.7)	10.3 (3.6–19.5)	10.0 (3.8–20.2)	7.5 (2.4–14.9)
Eosinophils (%)	0.0–6.0	4.8 (0.9–14.7)	5.3 (1.3–15.5)	6.2 (0.7–16.8)	6.3 (1.0–21.0)
Basophils (%)	0.0–2.0	1.7 (0.5–4.4)	2.0 (0.5–4.0)	1.7 (0.7–2.6)	1.4 (0.5–3.0)
Platelets (103/µL)	150–450	376 (157–538)	399 (167–644)	421 (136–705)	383 (157–659)
MPV (fL)	7.4–10.4	9.0 (7.2–13.3)	8.9 (7.4–11.8)	8.6 (6.3–11.7)	8.4 (7.1–10.8)
LDH (U/L)	336–618	1322 (775–2250)	1247 (743–1845)	1201 (665–1551)	1144 (703–1628)
Total bilirubin (mg/dL)	0.2 – 1.1	3.19 (1.5–5.9)	3.7 (1.6–7.4)	3.1 (1.2–6.4)	2.9 (1.4–7.8)
Indirect bilirubin (mg/dL)	–	3.1 (1.4–5.8)	3.6 (1.5–7.3)	3.0 (1.1–6.3)	2.8 (1.3–7.7)
Creatinine (mg/dL)	0.7–1.2	0.6 (0.4–1.0)	0.6 (0.3–0.9)	0.6 (0.3–0.9)	0.6 (0.4–0.9)
CRP (mg/dL)	0.0–0.8	1.3 (0.0–3.8)	0.9 (0.0–1.3)	1.3 (0.0–3.0)	3.3 (0.0–9.0)
ALAT (U/L)	50–120	33 (20–42)	38 (20–70)	34 (9–63)	41 (23–67)
Arginine (µmol/L)	94.2 ± 25.8*	45.2 (13.2–62.5)	46.0 (21.1–65.3)	46.6 (26.6–65.9) [d]	39.4 (16.4–60.4) [c]
Homoarginine (µmol/L)	1.50 ± 0.52**	1.18 (0.80–1.43)	1.20 (0.81–1.95)	1.18 (0.72–2.00)	1.14 (0.84–1.57)
ADMA (µmol/L)	0.42 ± 0.06*	0.75 (0.59–0.94)	0.76 (0.69–0.88)	0.78 (0.63–1.10)	0.74 (0.64–0.95)
SDMA (µmol/L)	0.47 ± 0.08*	0.55 (0.40–0.90)	0.56 (0.41–0.82)	0.54 (0.42–0.75)	0.53 (0.36–0.76)
ADMA/Arginine × 10^3	3.9–4.8***	20.8 (11.8–61.5)	19.44 (11.41–41.9)	18.26 (10.7–32.2)	21.8 (11.8–58.1)

Data are means (range) for 10 patients. Paired samples t- tests applying Bonferroni correction were used to determine whether there were significant differences within the different measurement points in all the measured parameters. [a], significantly different from baseline ($p<0.01$); [b], significantly different from 1 month ($p<0.01$); [c], significantly different from 2 months ($p<0.01$); [d], significantly different from 3 months ($p<0.01$). * Data from [48]; ** Data from [60]; *** Data from [13].

Abbreviations: ADMA, asymmetric dimethylarginine; ALAT, alanine aminotransferase; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBC, red blood cells; RDW, red blood cell distribution width; SDMA, symmetric dimethyl arginine; WBC, white blood cells.

Astaxanthin supplementation increased plasma- and RBC-astaxanthin levels

Both plasma- and RBC-astaxanthin increased from baseline (plasma mean: 33 nmol/L; RBC mean: 11 nmol/L packed RBC, respectively) to 1–3 months (plasma means: 225, 174, 167 nmol/L: RBC means: 149, 100, 71 nmol/L packed RBC) (Table 1). Figure 1 shows the courses of the mean plasma- (panel A) and RBC- (panel B) astaxanthin, together with the individual courses across the 3 months of supplementation.

Astaxanthin supplementation decreased reticulocytes, RDW, MCV and MCH

Hematological parameters are shown in Table 1. Reticulocyte percentages decreased from baseline to 3 months (9.5 to 5.6%) and from 2 to 3 months (8.8 to 5.6%). Concomitantly, also the RDW decreased from baseline to 3 months (19.2 to 16.7%) and from and 2 to 3 months (19.0 to 16.7%). The MCV decreased from 2 to 3 months (87.8 to 85.5 fL), and the MCH decreased from baseline to 3 months (29.8 to 28.0 pg). Figure 1 shows the courses of the RDW (panel C) and reticulocytes (panel D) across the 3-month astaxanthin supplementation period, together with the individual courses for the 10 patients. No significant differences were found between any of the other measured hematological and clinical chemical parameters across the different sampling points (Table 1).

Astaxanthin supplementation decreased plasma arginine but did not change homoarginine, ADMA, SDMA and the ADMA/arginine ratio

We found that the plasma arginine concentrations of the SCD patients (Table 1) were lower and ADMA levels higher, than the reference values that have previously been established with the same method ([48], Table 1). Current arginine levels were also lower than those of our own historical controls ([13] for subjects with HbAA (median 67; range 60–88 nmol/L)). On the other hand, the SDMA (Table 1; historical controls HbAA: median 0.33; range 0.33–0.35 nmol/L) and ADMA concentrations (HbAA: median 0.33; range 0.32–0.35 nmol/L) were higher in the current SCD study group. During astaxanthin supplementation, arginine levels decreased from 2 to 3 months (46.6 to 39.4 nmol/L), but no changes were found in either homoarginine, ADMA, SDMA or the ADMA/arginine ratio (Table 1).

Correlations between changes in reticulocytes and hematological parameters

The correlation between reticulocytes at baseline and the percentage change in reticulocytes from baseline to 3 months is shown in Figure 2A (p=0.038). The correlation indicates that the higher the baseline reticulocyte count, the higher percentage decrease in reticulocytes during astaxanthin supplementation. Reticulocyte count at baseline explained 27% of the reduction in reticulocytes from baseline to 3 months.

Positive correlations were found between the relative change in reticulocytes and the relative change in RDW (p=0.013) (Figure 2B) and LDH (p=0.033) (Figure 2D), indicating that the decreases in reticulocytes from baseline to 3 months corresponded with decreases in both RDW and LDH. Significant negative correlations were found between the relative change in reticulocytes from baseline to 3 months and the relative changes in RBC (p=0.020) (Figure 2C) and ALAT (p=0.032) (Figure 2E) in the same period, indicating that the decrease in reticulocytes corresponded with increases of both RBC and ALAT. The relative change in reticulocytes from baseline to 3 months explained 58, 80, 56 and 32% of the changes in RDW, RBC, LDH and ALAT, respectively. The correlation between the relative change in reticulocytes from baseline to 3 months and the relative change in the hematocrit during the same period did not reach significance (p=0.533) (Figure 2F).

Discussion

In this open label pilot study we supplemented 10 SCD patients in Dutch Sint Maarten with a daily oral dose of 8–12 mg astaxanthin during 3 months. The primary goal was to investigate whether astaxanthin incorporated into the RBC of these patients, and, secondary, whether this incorporation ameliorated the hemolytic component of the disease. Most importantly, we found that both plasma- and RBC-astaxanthin increased from baseline to 1–3 months (Table 1). Reticulocytes and RDW decreased from both baseline and 2 months to 3 months, MCV from 2 to 3 months and MCH from baseline to 2 months. There were, however, no changes in plasma ADMA concentrations. To our knowledge, this was the first time that astaxanthin supplementation was studied in SCD patients.

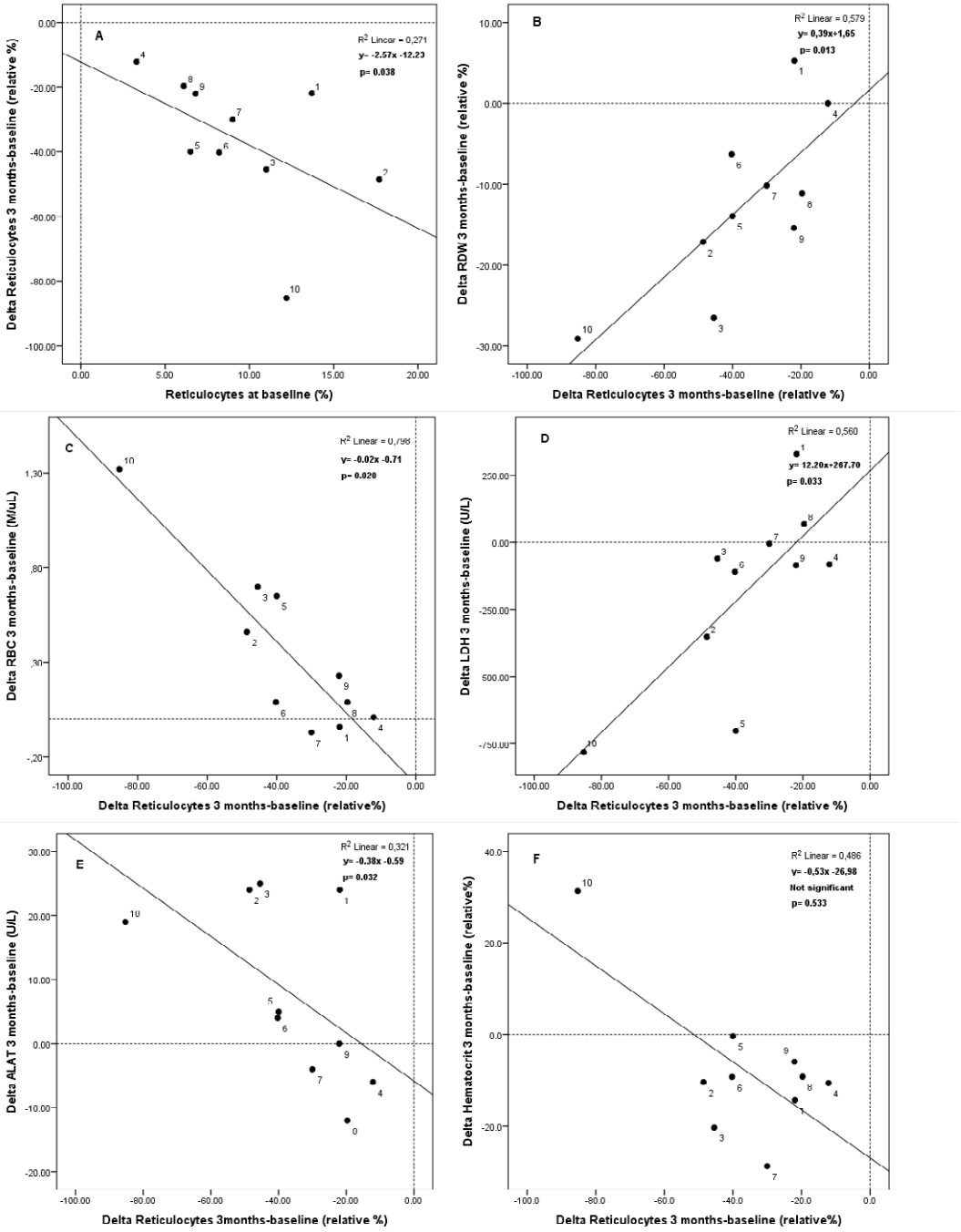

Figure 2. Correlations of reticulocytes at baseline or percentage changes in reticulocytes from baseline to 3 months with changes in hematological parameters in the same period

Panel A: Significant negative correlation of reticulocytes at baseline (in %) with the relative change (in %) of reticulocytes from baseline to

Astaxanthin kinetics and incorporation into RBC

We found that both plasma- and RBC-astaxanthin had increased after 1 month of astaxanthin supplementation (Figure 1, panels A and B), and decreased slightly thereafter until 3 months, although this decrease was not significant. The incorporation of astaxanthin in RBC confirms both the study of Nakagawa et al. [37] and our previous study, where we showed that after a single oral 40 mg dose, astaxanthin increases rapidly in the plasma of healthy subjects (peak value at 8 h) and somewhat later in their RBC (peak at 12 h). Subsequently, astaxanthin decreased rapidly in both plasma and RBC, with estimated half-lives of 18 and 28 h, respectively, suggesting that to reach RBC steady state levels, daily supplementation may be needed, at least until a whole body equilibrium is reached, if any [50]. Consistent with our previous study, we found that both plasma and RBC levels were subject to high intra- and inter-individual variations (Figure 1, panels A and B), which was attributed to non-standardized time difference between the last astaxanthin intake and blood sampling, fluctuating background astaxanthin intakes from the diet, variable bioavailability, large distribution volume, (induced) degradation, and possibly other factors [50].

In our previous study in healthy subjects with a single dose of 40 mg astaxanthin and a subsequent 8 mg maintenance dose during 17 days [50], we found that RBC contained medians of 44 and 49% from the plasma astaxanthin concentration, respectively. Also these data were subject to high intra- and inter-individual variations. These medians were, however, comparable with the estimated 43% calculated from the study of Nakagawa et al. [37], who supplemented healthy adults for 12 weeks with either 6 or 12 mg astaxanthin/day. In the present study with SCD patients, we found that the mean RBC concentration was 36% of the plasma concentration at baseline, but rose to almost double after 1 month supplementation (p=0.001) (Table 1). Whether the seemingly deviant baseline distribution of astaxanthin between plasma and RBC and the observed response of this distribution relates to the time of sampling after daily oral supplementation, the higher RBC turnover in SCD or other factors, should be studied in a larger population.

Effects of astaxanthin on the hemolytic component

SCD is characterized by chronic hemolysis, high reticulocyte counts [51] and low RBC half-lives (5–10 days; reference: 25–40 days) [2]. The observed modest decreases of reticulocytes and RDW from both baseline and 2 months to 3 months, the MCH from baseline to 3 months and the MCV from 2 to 3 months, suggests that RBC turnover diminished slightly, but notably in the last month of supplementation. A more detailed evaluation suggested that patients with the highest percentage reticulocytes at baseline might have benefited most from astaxanthin supplementation, as they presented the highest relative decrease in reticulocytes (Figure 2, panel A). This observation was consistent with the correlations found between the relative change in reticulocytes between 3 months and baseline and the changes across the same period in RDW (positive), RBC count (negative) and LDH (positive) (Figure 2, panels B, C and D, respectively), altogether suggesting that there was a slight decrease in RBC turnover notably after 2 months of supplementation.

Possible adverse effects

Somewhat unexpectedly, we found that the change in reticulocytes across the 3 months supplementation period correlated with an increase in ALAT,

3 months. The correlation suggests that patients with the highest reticulocyte percentages at baseline may have benefited most from astaxanthin supplementation. Panel B: Significant positive correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of the relative RDW (in %) in the same period. Panel C: Significant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of RBC counts (in 10^6/μL) in the same period. Panel D: Significant positive correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of LDH (in U/L) in the same period. Panel E: Significant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of ALAT (in U/L) in the same period. The correlation suggests that patients with the greatest relative decreases in reticulocyte percentages presented the highest increases in ALAT. Panel F: Insignificant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the relative change of the hematocrit (in %) in the same period. The insignificance of this relation suggests that the relative decrease of reticulocyte percentage was not accompanied with an increase of the hematocrit, which is an important determinant of the rheology. Abbreviations: ALAT, alanine aminotransferase; LDH, lactate dehydrogenase; RBC, red blood cells; RDW, red blood cell distribution width.

suggesting that patients with the highest decreases in reticulocytes exhibited the highest ALAT increases (Figure 2, panel E). Meanwhile, the group ALAT activity did not change, while all subjects presented ALAT values within the reference range (Table 1). We have at present no explanation for this suggested slight deterioration of liver function that came along with the decrease of the reticulocyte counts. No changes of ALAT activities have been reported in other astaxanthin supplementation studies. For instance, no changes in any transaminase were noted during the 12-week study of Nakagawa et al. [37] with either 6 or 12 mg astaxanthin/day.

Fortunately, the change of reticulocytes did not correlate significantly with a concomitant change of the hematocrit (Figure 2, panel F). Optimal oxygen transport efficiency for SCD patients occurs at a relatively low hematocrit. Sickle cells (either reversibly or irreversibly sickled cells) are intrinsically more rigid and viscous [52] and any augmentation of the hematocrit might be considered as an undesired effect.

We also found a decrease in arginine levels between 2 and 3 months of astaxanthin supplementation, though the levels at 3 months were not different from baseline. These changes did not cause any change in the ADMA/arginine ratio. Arginine is the substrate for NO synthesis, while ADMA inhibits NO formation. Consequently, any increase in the ADMA/arginine ratio, might reduce NO availability, causing a less favorable condition with less vasodilatation [12].

Limitations

Apart from the astaxanthin kinetics, none of the presently reported changes in hematological and clinical chemical parameters, either positive or negative, might be related to the supplementation, since the current study was not placebo controlled. It is for instance known that these parameters may vary with season, with e.g. variable exposure to infectious agents.

Conclusions

This open label pilot study showed that oral astaxanthin supplementation increases astaxanthin concentrations in both plasma and RBC of SCD patients without any observed adverse reactions. Most promising, we found a slight reduction of the reticulocyte count after 3 months, probably indicating a lower hemolysis; while many of the patients reported that they 'felt better'. Whether these effects are causally related to the intervention is worth being investigated in a larger randomized controlled intervention study, where astaxanthin would be provided at a similar or higher dose during a trial that preferably lasts for more than 3 months. It might be even better to include astaxanthin into a supplemental mix with other antioxidants (e.g. low dose vitamin E [53], beta-carotene, vitamin C and folic acid), minerals (selenium if necessary; and notably zinc; [54, 55]), amino acids (notably arginine [56, 57]), fish oil [53, 58] and vitamin D [59]. Antioxidants do not work on their own but are rather part of a yet poorly understood antioxidant network of free radical scavengers, quenchers and antioxidant enzymes and therefore, it seems improbable to find a single "magic bullet" to prevent or treat any disease associated with oxidative stress [20].

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CHAPTER 4

Higher prevalence of 'low T3 syndrome' in patients with chronic fatigue syndrome: A case-control study

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Abstract

Chronic fatigue syndrome (CFS) is a heterogeneous disease with unknown cause(s). CFS symptoms resemble a hypothyroid state, possibly secondary to chronic (low-grade) (metabolic) inflammation. We studied 98 CFS patients (21–69 years, 21 males) and 99 age- and sex-matched controls (19–65 years, 23 males). We measured parameters of thyroid function, (metabolic) inflammation, gut wall integrity and nutrients influencing thyroid function and/or inflammation. Most remarkably, CFS patients exhibited similar TSH, but lower FT3 (difference of medians 0.1%), TT4 (11.9%), TT3 (12.5%), %TT3 (4.7%), SPINA-GD (14.4%), SPINA-GT (14.9%), 24-hour urinary iodine (27.6%) and higher %rT3 (13.3%). FT3 below the reference range, consistent with the 'low T3 syndrome', was found in 16/98 CFS patients vs. 7/99 controls (OR 2.56; 95% CI=1.00–6.54). Most observations persisted in two sensitivity analyses with more stringent cut-off values for BMI, hsCRP and WBC. We found possible evidence of (chronic) low-grade metabolic inflammation (ferritin and HDL-C). FT3, TT3, TT4 and rT3 correlated positively with hsCRP in CFS patients and all subjects. TT3 and TT4 were positively related to hsCRP in controls. Low circulating T3 and the apparent shift from T3 to rT3 may reflect more severely depressed tissue T3 levels. The present findings might be in line with recent metabolomic studies pointing at a hypometabolic state. They resemble a mild form of 'non thyroidal illness syndrome' and 'low T3 syndrome' experienced by a subgroup of hypothyroid patients receiving T4 monotherapy. Our study needs confirmation and extension by others. If confirmed, trials with e.g. T3 and iodide supplements might be indicated.

Conflict of Interest and Funding DisclosurE

None declared.

Keywords

Chronic fatigue syndrome, thyroid, 'low T3 syndrome', triiodothyronine, reverse triiodothyronine, urinary iodine, inflammation, high sensitivity CRP.

Abbreviations

AA, arachidonic acid; AMC, Academic Medical Center; Anti-TG, anti-thyroglobulin antibodies; anti-TPO, anti-thyroid-peroxidase antibodies; CDR, cell danger response; CFS, chronic fatigue syndrome; CI, confidence interval; D2, deiodinase 2; D3, deiodinase 3; DHA, docoxahexaenoic acid; DNL, *de novo* lipogenesis; ELN, European Laboratory of Nutrients; EPA, eicosapentaenoic acid; FA, fatty acids; FT3, free triiodothyronine; FT4, free thyroxine; GD, sum activity of deiodinases; GT, secretory capacity of the thyroid gland; Hb, hemoglobin; HDL-C, High Density Lipoprotein-cholesterol; HPA, hypothalamus-pituitary-adrenal; HPT, hypothalamus-pituitary-thyroid; hsCRP, high-sensitive C-reactive protein; IL-1; interleukin-1; IL-6, interleukin-6; LPS, lipopolysaccharides; TNF, tumor-necrosis-factor; NFκB, nuclear factor kappa B; NTIS, non thyroidal illness syndrome; RBC, red blood cells; rT3, reverse T3; SPINA, structure parameter inference approach; sTSHi, standard TSH index; T2, 3,3'-diiodothyronine; TC, total cholesterol; TSH, thyrotropin; TT3, total triiodothyronine; TT4, total thyroxine; UMCG, University Medical Center Groningen; WBC, white blood cells.

Introduction

Chronic Fatigue Syndrome (CFS), also referred to as myalgic encephalomyelitis, is a complex heterogeneous disease, most commonly characterized by disabling fatigue, cognitive impairment, disrupted sleep and concomitant skeletal and muscular pain, lasting for more than six months and not improving with rest [1, 2] (for a broader definition, see [3]). Impaired physical and social functioning, vitality, emotional well-being and role limitations due to emotional problems [4] contribute to an impaired quality of life [5]. Although most patients have mild or moderate symptoms, some suffer from severe CFS and are housebound or even unable to move from their beds [4]. The diagnosis of CFS is based on the Fukuda criteria, i.e. symptoms, disability, and exclusion of explanatory illnesses, and not by means of physical signs or abnormalities in laboratory test results [1–3]. About 75% or more are female. The mean age of onset is 29–35 years and the mean illness duration ranges from 3–9 years [6], which implies that the symptoms are reversible. A meta-analysis of clinically confirmed cases in several countries indicates a prevalence of 0.76% [7]. In 2005, the prevalence of CFS in the Netherlands was slightly lower, 0.18–0.25% (30,000–40,000 patients among 16 million inhabitants) [8].

The underlying cause of CFS remains unclear. Many pathophysiological cascades have been hypothesized but underlying organic conditions are rarely found. Disturbed hypothalamus-pituitary-adrenal (HPA) axis, presented as mild hypocortisolism, heightened negative feedback and blunted responses to challenge have been found in CFS [9]. Computational analysis using endocrine and gene expression data suggest that CFS is associated with immune-mediated loss of thyroid function, exacerbated by a blunted HPA axis response [10]. Autonomic dysfunction, including orthostatic intolerance and syncope, microglial activation and structural changes, indicate involvement of the brain [11]. There is accumulating evidence that the cardiovascular system is compromised, with reports of autonomic dysfunction, attenuated heart rate and blood pressure [12] and increased death rate from heart failure [13]. The latter finding was related to a blunted cortisol response [14]. Taken together, dysfunctional central housekeeping involving interactions between both the HPA and HPT axes and the sympathetic/adrenal medulla, rather than single-hormone-axis disturbances, might play a key role in the development of CFS symptoms [10, 11, 14].

Dysregulation of the immune system in CFS may include autoimmune reactions and low-grade inflammation. Some studies demonstrated autoantibodies directed at diverse nuclear and neuronal components [15, 16] and against some neurotransmitters and neurotransmitter receptors in the CNS [17, 18]. Others associated infection and vaccination with later CFS onset [19, 20]. Recently, pandemic influenza A (H1N1) infection was related with a more than two-fold increased CFS risk [21]. A state of low-grade inflammation [22], as derived from elevated (hs)CRP [23], IL-6 [24], IL-1 and TNF-α [22] and/or NFκB [25] has, however, not consistently been found [26–28], possibly because of differences in experimental approaches and patient conditions [28]. Increased translocation of lipopolysaccharides (LPS) from Gram-negative enterobacteria with subsequent gut-derived inflammation was also found [29]. Giloteaux et al. demonstrated intestinal dysbiosis resulting from a more pro-inflammatory gut microbiome that may trigger the immune system [30]. Recently, the relationship between the thyroid with gut microbiome and inflammation became apparent from the associations of both hypothyroidism and levothyroxine use with small intestinal bacterial overgrowth [31].

Several symptoms resemble those of hypothyroidism. They are, however, not accompanied by the marked thyrotropin (TSH) increases of full-blown hypothyroidism [32]. Fuite et al. [10] suggested immune-mediated loss of thyroid function in CFS patients. Low-grade inflammation and subclinical hypothyroidism are not mutually exclusive. Inflammation virtually affects all hormonal axes [33], including the HPT axis [34]. Profound changes in this axis occur in the 'non thyroidal illness syndrome (NTIS)', also referred to as 'euthyroid sick syndrome', which has notably been investigated in critically ill patients [35]. As part of the acute phase response, this condition may reflect an adaptation to counteract excessive catabolism during severe illness [34]. The most important clinical chemical features of mild to moderate NTIS are normal/low-normal TSH, low total triiodothyronine (TT3) and free T3 (FT3) levels, normal/high-normal total thyroxine (TT4), decreased peripheral conversion of T4 to T3 and increased reverse T3 (rT3) levels [36]. Chronic inflammation in rodents increases the expression of deiodinase 3 (D3), which inactivates both T3 and T4 with concomitant production of 3,3'-diiodothyronine (T2) and rT3, respectively [34]. A recent study [37] also reported elevated concentrations of 3,5-T2 in humans affected by cardiac NTIS.

Chronic fatigue syndrome has been described as an ´allostatic overload condition´ [38], where the physiological mechanisms employed to deal with stress (also named 'allostatic states') contribute to the perpetuation of the disorder. CFS patients are 1.9 times more likely to have a high allostatic load index than healthy controls [39] and this allostatic load also correlates positively with CFS symptoms [40]. Thyroid allostasis-adaptive responses, presenting as NTIS, have been found in many conditions, ranging from critical illness, uremia and starvation to tumors [41]. Taken together, it is possible that, despite TSH and T4 levels within reference ranges, CFS symptoms may be attributable in part to allostatic responses, i.e. lower thyroid hormone activity, secondary to chronic (low-grade) inflammation caused by e.g. a compromised gut microbiome and gut wall integrity.

In the present case-control study, we focused on signs of low-grade inflammation and subclinical hypothyroidism. We measured parameters of thyroid function, low-grade inflammation and gut wall integrity [42], together with secondary markers of inflammation, also named metabolic inflammation [43, 44], including insulin resistance-mediated *de novo* lipogenesis (DNL), HDL-cholesterol (HDL-C), and the status of nutrients influencing thyroid function (iodine and selenium) and inflammation (fish oil fatty acids and vitamin D).

Materials and methods

Study design and study group

Patients were recruited in the Parkstad Clinic in Amsterdam, The Netherlands. They were diagnosed with CFS according to the CBO guideline [45]. These are based on the Fukuda criteria [1], with the exclusion criteria of Reeves [3]. In the Parkstad Clinic, 250 CFS patients are seen on a regular basis. From these, 150 were randomly selected to receive a letter requesting their voluntary participation. A total of 109 agreed to participate. Three of the participants were not patients of the Parkstad Clinic, making a total of 112 (see Figure 1 for flow scheme). The patients completed a questionnaire on their health, recent non-chronic medication use, smoking habits, supplement use and pregnancy and lactation. Exclusion criteria were use of medication that may affect thyroid function (e.g. T4, anti-arrhythmic drugs, such as amiodarone or corticosteroids), pregnancy, breastfeeding, and menstruation during urine collection. Other exclusion criteria were (biochemical) abnormalities that are excluded according to the CBO guideline and not demonstrated at the time of diagnosis, e.g. severe obesity (BMI >35 kg/m^2), infection (hsCRP >10 mg/L and white blood cells (WBC) >10*10^9/L), anemia [hemoglobin (Hb) <7.0 mmol/L in women and <8.0 mmol/L in men], hyperthyroidism (TSH below reference range with FT3 and/or free thyroxine (FT4) above reference range [46]), thyroid hormone resistance (elevated FT4 with non-suppressed TSH [47]), hypothyroidism (TSH above upper limit of reference range with FT4 below reference range) and subclinical hypothyroidism (TSH above reference range with normal FT4 [46]). Weights and lengths were measured on the spot. Data on age were obtained from interviews in the Dutch language.

A total of 119 age- and sex- matched apparently healthy controls were recruited by advertisement in the city of Groningen, The Netherlands. Health was self-reported with the aid of a health checklist filled out before inclusion. Primary exclusion criteria were the use of any chronic medication, menstruation during urine collection, severe obesity (BMI>35 kg/m^2), and both pregnancy and breastfeeding. Incidental use of analgesics and short-term medication (e.g. antibiotics, more than four weeks ago) were allowed. Secondary exclusion criteria were infection (hsCRP>10 mg/L and WBC>10*10^9/L), anemia [Hb <7.0 mmol/L in women and <8.0 mmol/L in men], hyperthyroidism (TSH below reference range with FT3 and/or FT4 above reference range [46]), thyroid hormone resistance (elevated FT4 with non-suppressed TSH [47]), hypothyroidism (TSH above upper limit of reference range with FT4 below reference range) and subclinical hypothyroidism (TSH above reference range with normal FT4 [46]). Data on age were obtained from interviews in the Dutch language. Weight and height were self-reported.

All patients and controls received a verbal and written explanation of the objectives and procedures and all provided us with written informed consent. The study was in agreement with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The protocol was approved by the University Medical Center Groningen (UMCG) Medical Ethical Committee (NL44299.042.13, METc 2013/154, dated 12th of august, 2013).

Sample size and final study groups

The calculation of the sample size (i.e. 100 subjects per group) was based on the correlation coefficient

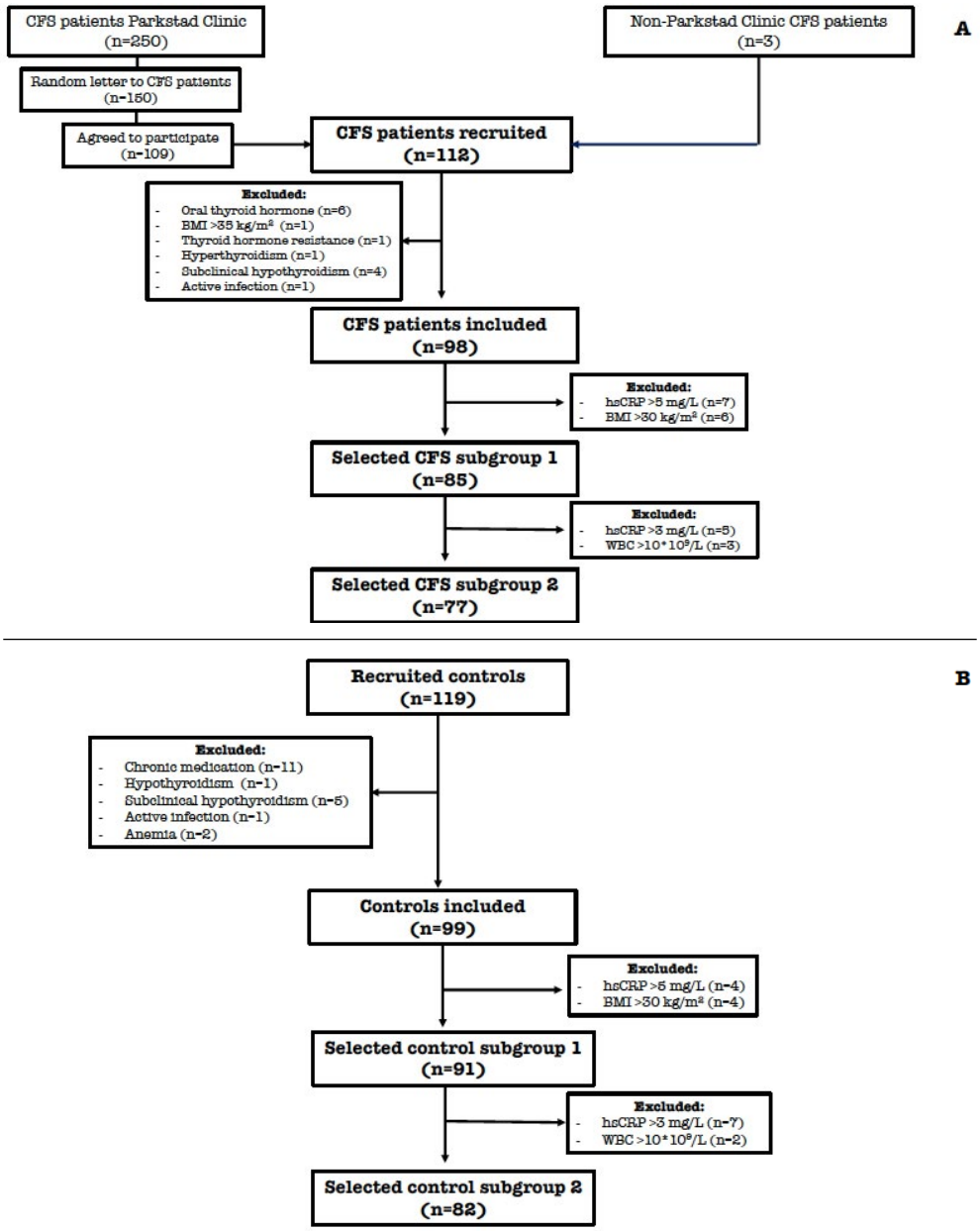

Figure 1. Flow-chart: inclusion of chronic fatigue syndrome patients (A) and controls (B) in the different groups and subgroups.
Abbreviations: CFS, chronic fatigue syndrome; n = number of subjects; BMI, body mass index; hsCRP, high-sensitive C-reactive protein; WBC, white blood cells.

of a comparable population using different steps (for more information, see [48]). For this, we used the correlation coefficient found by Girvent et al. [49] for the association of both inflammatory markers CRP and IL-6 with rT3, choosing the highest (i.e. r=0.75 for rT3 vs. CRP). In this study, subjects with NTIS were

compared with patients without euthyroid sick syndrome, both undergoing surgery. Assuming a 95% confidence interval (CI) of [0.59, 0.79], we estimated the sample size using IBM SPSS Statistics (version 20), with the obtained formula, where the n (sample size) appeared inside the Euler number exponent (e). We anticipated 20% exclusion based on abnormal laboratory data, and therefore aimed at the initial inclusion of 120 patients and controls.

We gathered information about supplement intake (vitamin D and fish oil) from 71/98 CFS patients. Users were defined as supplementing themselves either with multivitamins and/or other supplements containing that specific nutrient.

Subsequently, we performed a sensitivity analysis applying stricter exclusion criteria for possible signs of (low-grade) inflammation ('selected groups 1 and 2', see Results and Figure 1). In the first sensitivity analysis, both CFS patients and controls with BMI >30 kg/m^2 and/or hsCRP >5 mg/L were excluded. In the second one, we also excluded subjects with hsCRP >3 mg/L and/or with WBC >10*10^9/L.

Sample collection and analyses

Approximately 50 mL of blood were collected by venipuncture in the non-fasting state in three types of tubes (EDTA anticoagulated, lithium-heparin anticoagulated and serum separator). Samples were processed within 2 h after collection. Twenty-four-hour urine samples were collected and their volumes measured. Samples were stored at –20 °C and sent to the participating laboratories [UMCG, laboratory of Special Chemistry and Radiochemistry, Academic Medical Center in Amsterdam (AMC), Medical Laboratories, Reinier de Graaf Groep Diagnostisch Centrum, Delft and European Laboratory of Nutrients (ELN), Bunnik].

EDTA-whole blood was used for the measurement of routine hematological parameters [Hb, hematocrit, WBC, red blood cells (RBC) and thrombocytes] with a Sysmex XN-9000 Hematology Analyzer (Sysmex Nederland BV, Etten Leur, The Netherlands). The remainder of the EDTA blood was separated into thrombocyte-rich plasma and an RBC pellet by centrifugation for 10 min at 1,800 g . RBC were washed three times with 0.9% NaCl and re-suspended to an about 50% hematocrit. After washing, 200 µl of the RBC suspension was transferred to a teflon-sealable 'Sovirel' tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0 (internal standard). In this ready-to-transmethylate mixture, fatty acids (FA) are stable at room temperature and in the dark for months [50]. After centrifugation (10 min, 1,800 g) of the thrombocyte-rich EDTA-plasma, we aliquoted the isolated thrombocyte-poor EDTA plasma and stored it in 2 mL cryovials at –20 °C. Lithium-heparin whole blood (1.5 mL) was aliquoted for measurement of elements. The remainders of the lithium-heparin anticoagulated blood and the coagulated blood sample were centrifuged for 10 min at 1,800 g. The resulting plasma and serum were isolated, transferred to 2 mL cryovials and stored at –20 °C until analysis.

RBC-FA compositions were determined by capillary gas chromatography/flame ionization detection in the UMCG, using previously described procedures [50]. RBC-FA contents were expressed in g/100 g FA (g%). Tryptophan and kynurenine were measured in EDTA-plasma by LC–electrospray ionization–MS/MS as previously described [51]. Serum 25(OH)D2 and 25(OH)D3 [together referred to as 25(OH)D)] were determined by isotope dilution-online solid-phase extraction liquid chromatography–tandem mass spectrometry (ID-XLC-MS/MS) in the UMCG [52]. Plasma MMA was measured by LC-MS/MS according to Nelson et al. [53]. Serum iron, ferritin, hsCRP, total cholesterol (TC) and LDL- and HDL-cholesterol were measured using a Roche Modular P module (Roche, Almere, The Netherlands). Vitamin B12, folate, TSH, FT4, and FT3 were assayed by electrochemiluminescent immunoassay on the Roche Modular E170 Analyzer. Serum TT4 and TT3 were measured using an Architect i2000SR (Abbott Diagnostics, Hoofddorp, The Netherlands). Serum anti-thyroglobulin antibodies (anti-TG) and anti-thyroid-peroxidase antibodies (anti-TPO) were measured with an Immulite 2000 (Siemens, The Netherlands). Plasma rT3 was measured by in-house RIA [54] at the AMC Amsterdam, The Netherlands. Plasma homocysteine was analyzed in the UMCG by competitive protein binding assays with the use of an immunochemistry analyzer (IMX; Abbott Diagnostics, Hoofddorp, The Netherlands).

Whole blood- and lithium-heparin plasma selenium, copper, magnesium and zinc and iodine in urine were measured using ICP-MS 7700x (Agilent, Amstelveen The Netherlands) in the ELN. Selenium, copper, magnesium and zinc contents in RBC were calculated from their concentrations in plasma and whole blood, using hematocrit values for correction. Plasma zonulin (active form) concentrations were measured using the K5600 ELISA kit (Immundiagnostik AG, Bensheim,

Germany). The quantification of 8-iso-prostaglandin F2-isoprostanes in urine was performed by GC-tandem-MS using a two-step derivatization and a selective solid-phase extraction protocol with HLB and Silica columns as described by Zhao et al. [55]. The tryptophan/kynurenine ratio was calculated. This ratio may be decreased during inflammation [56, 57].

For the investigation of the pathogenesis of the ´low-T3 syndrome´, we measured FT3/FT4, TT3/TT4 and rT3/TT3 ratios. For the investigation of the underlying etiology of the ´low-T3 syndrome´, we calculated the following variables of thyroid metabolism: standard TSH index (sTSHi), in order to quantify the thyrotropic function of the pituitary [58]; the sum activity of deiodinases (structure parameter inference approach (SPINA)-GD) as a variable for deiodination function [59]; the secretory capacity of the thyroid gland (SPINA-GT), as an evaluation of thyroid secretory status [59]; and the ratios of TT3/FT3 and TT4/FT4 as evaluations of protein binding of thyroid hormones. The sTSHi was calculated as TSHi = (TSH – 2.70) / 0.676 [58]. SPINA-GD and -GT were calculated as SPINA-GD = $[\beta_{31} \times (K_{M1} + FT4) \times TT3] / (\alpha_{31} \times FT4)$ and SPINA-GT = $\beta_T \times (D_T + TSH) \times TT4] / (\alpha_T \times TSH)$. Constants in the equations were as follows: $\beta_{31} = 8 \times 10^{-6}$/s, $K_{M1} = 5 \times 10^{-7}$ mol/L, $\alpha_{31} = 0.026$/L, $\beta_T = 1.1 \times 10^{-6}$/s, $D_T = 2.75$ mU/L, and $\alpha_T = 0.1$/L [59, 60]. The rT3/TT3 ratio was also calculated as a proxy for a metabolic shift. For the latter, we also calculated the %TT4, %TT3 and %rT3 by dividing their concentrations by the sum of TT4+TT3+rT3 and adjusting to 100%. Zinc/copper, TC/HDL-C and eicosapentaenoic (EPA)/ arachidonic acid (AA) ratios were also calculated. A proxy for hepatic DNL (DNL liver) was calculated according to Kuipers et al. [61] (sum of RBC 16:0, 16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9). The omega-3 index, RBC-EPA+ docosahexaenoic acid (DHA) (RBC-EPA+DHA) was calculated.

Statistics

Statistical analyses were performed with IBM SPSS Statistics 23 SPSS Inc, Chicago, IL. Mann-Whitney U tests were used for the evaluation of between-group differences in the distribution. The Chi-Square tests were used for the evaluation of between-group differences in nominal variables. Odds ratios were calculated to quantify the strength of the presence of low T3 in the different groups. Correlation analyses were performed using Spearman's Rho for non-parametric variables.

Results

Of the 112 initially included CFS patients, six taking oral thyroid hormone and one with BMI >35 kg/m^2 were excluded, leaving 105 patients. Of these, one subject with thyroid hormone resistance (defined as elevated serum levels of FT4 with non-suppressed TSH [47]), one with hyperthyroidism (TSH below reference range with FT3 and/or FT4 above reference range [46]), four with subclinical hypothyroidism (TSH above reference range with normal FT4 [46]) and one suspected of active infection (both hsCRP >10 mg/L and WBC >10*10^9/L) were excluded; making a total of 98 finally included CFS patients (Figure 1).

Of the 119 age- and sex-matched apparently healthy controls, 11 taking chronic medication were excluded, leaving 108 controls. Of these, one with hypothyroidism (TSH above reference range with FT4 below reference range), five with subclinical hypothyroidism (TSH above reference range with normal FT4 [46]), one suspected of active infection (both hsCRP >10 mg/L and WBC >10*10^9/L) and two with anemia were excluded; making a total of 99 finally included healthy controls (Figure 1).

Whole study group

Characteristics of the 98 CFS patients and the 99 controls are shown in Table 1. The CFS patients (21 males, 77 females) had a median age of 43 years (range 21–69), median height of 172 cm (149–198), median weight of 68 kg (48–118) and median BMI of 22 kg/m^2 (18–34). The age-and-sex-matched healthy controls (23 males, 76 females) had a median age of 39 years (19–65), median height of 173 cm (156–193), median weight of 70 kg (47–100) and a median BMI of 23 kg/m^2 (18–33). The above anthropometric characteristics exhibited no between-group differences.

Thyroid hormones

CFS patients exhibited lower FT3, TT4, TT3, %TT3, SPINA-GD and SPINA-GT, lower ratios of TT3/TT4, FT3/FT4, TT3/FT3, and TT4/FT4; and higher %rT3 and rT3/TT3 ratio. There were no between-group differences in other thyroid hormone parameters, notably TSH, FT4, rT3 and %TT4, (Table 1). FT3 below the reference range was more frequently found in CFS patients (16/98) as compared to controls (7/99; p=0.035) with an odds ratio of 2.56 (95% CI=1.00–6.54).

Table 1. Anthropometrics and laboratory data of 98 CFS patients and 99 controls.

			CFS Patients	Controls			CFS Patients		Controls	
		Units	Median (range)	Median (range)	p value	Reference range/cut-off value	% (n) below	% (n) above	% (n) below	% (n) above
Anthropometrics	Number		98	99						
	Gender	male/female	21/77	23/76						
	Age	years	43 (21–69)	39 (19–65)	0.235					
	Height	cm	172 (149–198)	173 (156–193)	0.996					
	Weight	kg	68 (48–118)	70 (47–100)	0.618					
	BMI	kg/m²	22 (18–34)	23 (18–33)	0.384	<30	–	9 (9)	–	4 (4)
Thyroid function	TSH	mU/L	1.43 (0.49–4.40)	1.59 (0.53–3.32)	0.527	0.5–4	1 (1)	1 (1)	0 (0)	0 (0)
	FT4	pmol/L	15.9 (11.4–23.0)	15.6 (11.0–19.7)	0.562	11.0–19.5	0 (0)	5 (5)	0 (0)	1 (1)
	FT3	pmol/L	5.2 (3.9–6.9)	5.2 (3.2–12.8)	0.047 *	4.4–6.7	16 (16)	2 (2)	7 (7)	17 (17)
	TT4	nmol/L	63.4 (17.8–121.3)	72.0 (45.4–134.8)	<0.001 **					
	TT3	nmol/L	1.4 (0.4–2.5)	1.6 (1.2 –2.3)	<0.001 **					
	rT3	nmol/L	0.23 (0.08–0.40)	0.23 (0.12–0.41)	0.783	0.11–0.44	1 (1)	0 (0)	0 (0)	0 (0)
	% TT4		97.55 (96.69–98.44)	97.55 (96.61–98.47)	0.513					
	% TT3		2.04 (1.21–2.94)	2.14 (1.24–3.12)	0.012 *					
	% rT3		0.34 (0.12–1.14)	0.30 (0.15–0.45)	<0.001 **					
	TT3/TT4 ratio	mmol/mol	21.0 (12.3–30.4)	21.93 (12.62–32.26)	0.013 *					
	FT3/FT4 ratio	mol/mol	0.32 (0.20–0.49)	0.34 (0.24–0.74)	0.004 **					
	rT3/TT3 ratio	mol/mol	0.18 (0.05 –0.60)	0.15 (0.08–0.24)	<0.001 **					
	TT3/FT3 ratio	mol/mmol	0.28 (0.08–0.42)	0.31 (0.13–0.45)	<0.001 **					
	TT4/FT4 ratio	mol/mmol	4.08 (1.26–6.84)	4.62 (3.15–9.05)	<0.001 **					
	SPINA–GT	pmol/s	1.77 (0.37–4.36)	2.08 (1.07–6.43)	0.010 *					
	SPINA–GD	nmol/s	13.42 (4.36–23.89)	15.67 (10.15–25.05)	<0.001 **					
	sTSHi		–1.89 (–3.27–2.51)	–1.65 (–3.21–0.92)	0.527					
Inflammation	WBC	10^9/L	6.1 (3.3–11.7)	6.3 (3.7– 12.0)	0.182	4–10	5 (5)	5 (5)	3 (3)	5 (5)
	hsCRP	mg/L	0.94 (0.09–8.28)	0.77 (0.11–21.62)	0.254	<5.0	–	7 (7)	–	4 (4)
	Kynurenine	µmol/L	1.63 (0.79–2.97)	1.81 (0.94–3.03)	0.001 **	1.14–3.02	14 (14)	0 (0)	3 (3)	1 (1)
	Tryptophan	µmol/L	54.0 (27.9–88.7)	56.4 (30.9–98.6)	0.003 **	45–83	19 (19)	1 (1)	3 (3)	2 (2)
	Tryptophan/Kynurenine	mol/mol	32.57 (18.47–63.78)	32.42 (17.43–56.92)	0.443					
	Ferritin [1]	µg/L	77 (8–600)	52 (5–386)	0.007 *	Men 30–400	1 (1)	1 (1)	0 (0)	0 (0)
						Women 15–130	1 (1)	10 (10)	12 (12)	13 (13)
	Urinary isoprostanes	nmol/d	1271 (164–6830)	1336 (170–9978)	0.373					
	TC	mmol/L	5.2 (2.8–7.6)	5.1 (3.0–9.1)	0.627					
	HDL–C [2]	mmol/L	1.4 (0.6–3.9)	1.6 (0.7–3.2)	<0.001 **					
	LDL–C	mmol/L	3.1 (1.1–5.6)	3.1 (1.1–7.0)	0.792					
	TC/HDL–C [2]	mol/mol	3.5 (1.7–10.7)	3.1 (1.7–9.0)	0.001 **					
	DNL Liver	g%	34.98 (32.37–43.29) [3]	36.26 (33.58–43.85)	<0.001 **					
Intestinal permeability	Zonulin	ng/mL	1.24 (0.17–2.27)	1.39 (0.25–2.89)	0.002 **					
	Nutritional factors									
	Urinary Iodine (24 h)	µg/d	113 (20–559)	156 (27–666)	<0.001 **	> 200	87 (85)	11 (11)	66 (65)	22 (22)
	Selenium (P)	mg/L	0.08 (0.05–0.27)	0.09 (0.06–0.46)	0.103	0.08–0.30	42 (42)	0 (0)	32 (32)	1 (1)
	Selenium (IC)	mg/L	0.17 (0.11–0.97)	0.15 (0.04–0.31)	0.001 **	0.17–0.55	45 (44)	2 (2)	62 (61)	0 (0)
	25 (OH) Vitamin D	nmol/L	75.8 (16.0–217.2)	54.9 (5.4–133.4)	<0.001 **	80–250	59 (58)	0 (0)	83 (82)	0 (0)
	RBC–EPA+DHA	g%	4.08 (1.95–7.81)	4.07 (1.91–8.54)	0.884	>8	100 (99)	0 (0)	98 (97)	2 (2)
	RBC EPA/AA	g%	0.04 (0.01–1.00)	0.04 (0.01–0.18)	0.288					

(Metabolic) inflammation

We did not find significant differences in WBC, hsCRP, tryptophan/kynurenine ratio and urinary isoprostanes. CFS patients displayed lower kynurenine and tryptophan, as compared to the healthy controls. Taking both genders together, we found that ferritin was higher in CFS patients as compared to controls. Analyzed according to gender, we found that ferritin was higher in both male and female CFS patients, as compared to their apparently healthy counterparts (females: 77 CFS vs. 76 controls; p=0.003; males 21 CFS patients vs 23 controls; p=0.012) (data not shown in Table 1). Taking both genders together, we found that HDL-C was lower and the TC/HDL-C ratio higher in CFS patients as compared to controls. Analyzed according to gender, we found that HDL-C was lower in both male and female CFS patients, as compared to their apparently healthy counterparts (females: 77 CFS vs. 76 controls; p<0.001; males 21 CFS patients vs 23 controls; p=0.04). The TC/HDL-C ratio was higher in female CFS patients compared to controls (p=0.001) data not shown in Table 1). The RBC-FA composition showed a lower hepatic DNL in CFS patients.

Zonulin, a parameter of intestinal permeability [42], was lower in CFS patients as compared to controls.

Nutritional factors influencing thyroid function and inflammation

The 24-hour urinary iodine output, as a proxy of iodine status, was lower in CFS patients. Plasma selenium was similar, but intracellular selenium was higher (Table 1).

Vitamin D [25(OH)D] status of CFS patients was higher. Nevertheless, 58 patients (59%) and 82 (83%) controls presented 25(OH)D levels below the optimal cut-off of 80 nmol/L. None of the patients and 2% of the controls exhibited RBC-EPA+DHA contents above 8 g%, which is considered to confer optimal protection against cardiovascular [62] and neuropsychiatric [63] diseases. CFS patients and controls exhibited no differences in RBC-EPA/AA ratio, which is a risk factor for cardiovascular disease [64] and inflammation-induced depression [65]. We gathered information about supplement intake from 71/98 CFS patients. Among these, the users did not exhibit higher status of vitamin D (plasma 25(OH)D; 30 vs. 41; p=0.48), or EPA+ DHA (RBC–EPA+DHA; 7 vs. 64; p=0.44).

Additional laboratory data, including hematological indices, nutrient status influencing anemia, and other RBC-fatty acids can be found in Supplementary Table 1.

Interim conclusions based on the whole group

Figure 2 shows the case-control between-group differences in terms of percentages lower or higher of the medians of CFS patients compared to those of controls. Only significant between-group differences are depicted. Most remarkably, the results for the whole group indicated similar TSH in patients with CFS, but subtle changes in thyroid hormone concentrations, with an apparent shift in their metabolism. CFS patients notably exhibited relatively lower FT3, TT4, and TT3; lower deiodination function (SPINA-GD), lower thyroid secretory function (SPINA-GT), lower protein binding of thyroid hormones (TT3/FT3, TT4/FT4), and lower T3/T4 hormone ratios (TT3/TT4, FT3/FT4), lower %TT3, higher %rT3, and higher rT3/TT3 ratio. The lower 24-hour urine iodine output of CFS patients was also remarkable.

Sensitivity analyses

The strength of the above findings for the whole group was tested with 'sensitivity analyses'. For this goal, we created 'selected subgroups 1 and 2' by exclusion of subjects with the most prominent signs of (metabolic) inflammation, defined as relatively high hsCRP and BMI.

Table 1. Anthropometrics and laboratory data of 98 CFS patients and 99 controls — continued.

Data are medians (ranges). Mann-Whitney U tests were used for between-group differences in the distribution.[1] When analyzed according to gender, ferritin was higher in both male and female CFS patients as compared to controls (data not shown). [2] When the TC/HDL-C ratio and HDL-C were evaluated according to gender, we did not find differences in the TC/HDL-C ratio in the relatively small number of men, but those in the females persisted. HDL-C was lower in both male and female CFS patients compared to controls (data not shown). * Significant at p<0.05. ** Significant at p<0.01. Abbreviations: WBC, white blood cells; RBC, red blood cells; hsCRP, high-sensitive C-reactive protein; P, plasma; IC, intracellular; TSH, thyrotropin; FT4, free thyroxin; FT3, free triiodothyronine; TT4, total thyroxin; TT3, total triiodothyronine; rT3, reverse T3; GD, sum activity of deiodinases; GT, secretory capacity of the thyroid gland; sTSHi, standard TSH index; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DNL, *de novo* lipogenesis, sum of RBC 16:0,16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9, according to [61].

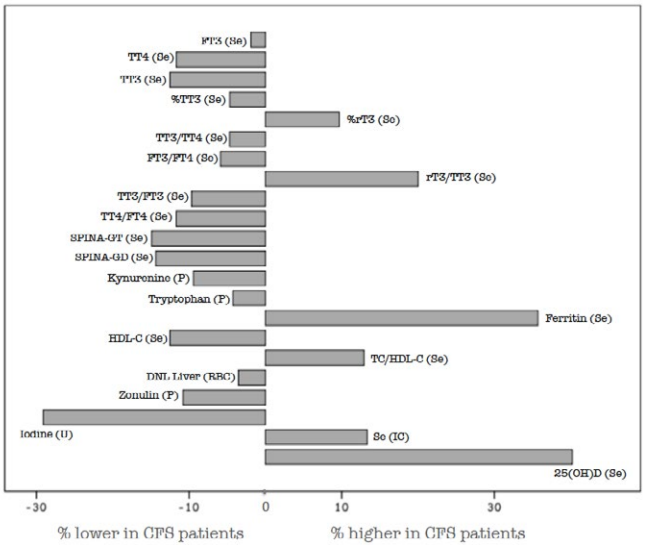

Figure 2. Between-group differences in parameters, depicted as percentages relative to control.

Only parameters exhibiting significant between-group differences are depicted (see Table 1). Data are calculated from the medians (Table 1) according to ((median CFS-median controls)/median controls*100) (in %). Abbreviations: Se, serum; P, plasma; IC, intracellular; U, urinary; FT4, free thyroxin; FT3, free T3; TT4, total T4; TT3, total T3; rT3, reverse T3; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; DNL, de novo lipogenesis, sum of 16:0,16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9, according to [61]; SPINA-GD, sum activity of deiodinases; SPINA-GT, secretory capacity of the thyroid gland; sTSHi, standard TSH index.

Selected subgroups 1

Of the 98 CFS patients, seven with hsCRP >5 mg/L (of whom three had BMI>30 kg/m^2) and six others with a BMI >30 kg/m^2 were excluded to create a subgroup of 85 patients (selected CFS subgroup 1) (Figure 1). Of the 99 healthy controls, four with hsCRP >5 mg/L and four others with a BMI >30 kg/m^2 were excluded to create a subgroup of 91 controls (selected control subgroup 1) (Figure 1). All parameters of thyroid function and inflammation that were significantly different between CFS patients and controls in the whole group, remained significant after this sensitivity analysis.

Selected subgroups 2

Of the 85 CFS patients in selected subgroup 1, five with hsCRP >3 mg/L and three with WBC >10*10^9/L (as more sensitive markers of inflammation) were excluded to create a subgroup of 77 patients (selected CFS subgroup 2) (Figure 1). Of the 91 controls in selected subgroup 1, seven subjects with hsCRP >3 mg/L and two with WBC >10*10^9/L were excluded to create a subgroup of 82 controls (selected control subgroup 2) (Figure 1). Characteristics of these CFS patients and controls, together with their clinical chemical data are shown in Supplemental Table 2. FT3 below the reference range was more frequently found in CFS patients (16/77) as compared to controls (7/82; p=0.024) with an odds ratio of 2.81 (95% CI=1.09–7.27), although the FT3 was no longer significantly lower. The higher ferritin proved no longer significantly different (compared to sensitivity analysis 1). However, ferritin remained higher in male and female CFS patients as compared to their apparently healthy counterparts (females: 59 CFS vs. 60 controls; p=0.015; males 18 CFS patients vs 22 controls; p=0.026) (data not shown in Supplemental Table 2). Analyzed according to gender, we found that HDL-C remained lower and the TC/HDL-C ratio remained higher in female CFS patients as compared to the healthy controls (females: 59 CFS vs. 60 controls; p=0.010 and p=0.007) (data not shown in Supplemental Table 2).

Conclusions based on the sensitivity analyses

Most importantly, we found that most of the subtle between-group thyroid hormone differences persisted when CFS patients and controls with more signs of (metabolic) low-grade inflammation were excluded, except for the occurrence of lower FT3 in CFS patients. However, FT3 below the reference range remained more frequent in CFS patients after applying stricter exclusion criteria.

Correlation analysis

We found that FT3, TT3, TT4 and rT3 were positively related to hsCRP in the CFS patients (n=98) and the combined controls and CFS patients (n=197) (Supplemental Figure 1). TT3 and TT4 were also positively related to hsCRP in the controls (n=99) (Supplemental Table 3). TSH and FT4 did not correlate with hsCRP.

When combined, controls and CFS patients with low FT3 (<4.4 pmol/L) were also found to more often exhibit low hsCRP (<1 mg/L) in the whole group (p=0.001) (n=197; OR 6.22; 95% CI=1.78–21.70) and in selected group 2 (p=0.011) (n=159; OR 4.26; 95% CI=1.20–15.03).

Discussion

The most remarkable observation in this case-control study was that, as a group, the present CFS patients exhibited lower FT3, TT4, TT3, %TT3, SPINA-GD, SPINA-GT, T3/T4 ratios, lower protein binding of thyroid hormones and 24-hour urinary iodine excretion, together with higher %rT3. Sixteen (16%) CFS patients exhibited the 'low T3 syndrome' as compared to seven (7%) controls (Table 1). These observations were basically unaltered upon applying more stringent cut-off values for hCRP, BMI and WBC in our 'sensitivity analyses' (Supplemental Table 2). CFS patients also showed some signs of (metabolic) low-grade inflammation, notably higher TC/HDL-C and ferritin, and lower HDL-C, tryptophan and kynurenine. When the TC/HDL-C ratio, HDL-C and ferritin were evaluated according to gender, we did not find differences in the TC/HDL-C ratio in the relatively small number of men (n=21), but those in the females (n=77) persisted. HDL-C was lower and ferritin remained higher in both male and female CFS patients compared to controls. Therefore, we conclude that, in the present study, we found subtle evidence of low-grade (metabolic) inflammation in CFS patients. Plasma 25(OH)D below the optimal cut-off value of 80 nmol/L was found in 59% of the CFS patients and 83% of controls. Both CFS patients and controls exhibited low fish intakes, as reflected by their low omega-3 index of about 4.1 g%. An omega-3 index of 8 g% is considered to confer optimal protection against cardiovascular [62] and neuropsychiatric diseases [63].

Comparison with Non Thyroidal Illness Syndrome (NTIS)

The 'low T3 syndrome' encountered in a subgroup of CFS patients bears clinical chemical similarity with NTIS features. Both syndromes are biochemically characterized by low TT3 and FT3 together with normal/high-normal FT4 and normal TSH, at least in the mild and moderate forms of NTIS [36]. The clinical disparity relates to the underlying severity of the diseases that are usually linked to NTIS, as opposed to the chronicity and less life-threatening nature of CFS [66]. NTIS is a typical feature of critically ill patients in intensive care units, although similar changes in the HPT-axis have been observed during calorie restriction and in patients with non-critical chronic diseases, such as heart failure, chronic obstructive pulmonary disease and diabetes mellitus [67], also referred to as mild, or atypical forms of NTIS [36, 67]. All of these conditions, especially calorie restriction, might find a common denominator in an adaptive response aiming at saving energy and body protein in order to outstay any potential acute stress stimulus [68–70]. Through coordinated changes in thyroid hormone metabolism, transport and receptors, NTIS might mechanistically reflect a cytokine-orchestrated allostatic condition that is remote from the well-known homeostatic negative feedback regulation of the HPT axis [71].

Low T3 syndrome might be in line with recent data of the CFS epigenome and metabolome

A recent study identified 12,608 differentially methylated sites in peripheral blood mononuclear cells of 49 female CFS patients vs. 25 healthy female controls. These sites were predominantly involved in metabolism and to a lesser extent in neuronal cell development. Among these sites, 1,600 were related to a score of self-reported quality of life, while 13 sites were associated with glucocorticoid sensitivity in a subgroup of CFS patients with glucocorticoid hypersensitivity [72]. In line with downregulated energy expenditure, recent CFS case-control studies of the metabolome revealed abnormalities in several metabolic pathways, including those reflecting mitochondrial metabolism, consistent with a hypometabolic state [73, 74]. The lower proxy for DNL encountered in the currently studied CFS patients might fit into this picture, since hypothyroidism in mice has been shown to downregulate the rate limiting enzymes involved in DNL [75]. In addition, induced hypothyroidism in humans for two weeks causes profound changes in FA metabolism [76]. Another recent case-control study using metabolic profiling showed an altered serum amino acid profile in CFS patients, suggesting impaired mitochondrial pyruvate oxidation [74], a condition likely to reflect energy deficiency and excessive lactate production, with utilization of amino acids from endogenous protein as alternative TCA cycle substrate. The 'low T3 syndrome' in a subgroup of CFS patients found in this study might be cause and consequence of the above noted epigenetic changes [72] and a driving force of the metabolic differences noted

by others [73, 74] and by us. Through both genomic and non-genomic actions, T3 has profound impacts on mitochondria and metabolism [77], including several pathways regulating the expression of target genes contributing to mitochondrial biogenesis [78].

Correlation of thyroid hormones with hsCRP

The association between low T3 and low hsCRP suggests that both CFS patients and controls with low FT3 are less responsive to inflammatory stimuli, which is in line with observations by others. In apparently healthy individuals, Hodkinson et al. [79] found, amongst others, that TT3 concentrations are positively related to monocyte phagocytic activity and expression of interleukin-6 (IL-6) by activated monocytes, while TT4 is positively related to CRP. Their data suggest that higher thyroid hormone concentrations within the normal range enhance innate and adaptive immunity by greater responsiveness to immune stimuli. Accordingly, Rozing et al. [80] showed that, although higher circulating levels of inflammatory markers were associated with lower levels of free serum FT3; higher serum FT3, but not higher TSH and FT4, are related to a higher production capacity of pro-inflammatory cytokines (IL-1β, IL-6, TNF-alpha) in whole blood of 85 year-old women and men, following *ex vivo* stimulation with LPS. They suggest a mutual association between T3 and pro-inflammatory cytokines, whereas T3 stimulates production of pro-inflammatory cytokines that subsequently diminish the conversion of T4 to T3. Finally, evidence of a diminished specific immune response has been found in patients with CFS. Investigating pokeweed mitogen-stimulated isolated peripheral blood mononuclear cells, Loebel et al. [81] found a deficient EBV-specific immune response in patients with CFS, possibly causing impaired EBV control. Taken together, it is possible that a subgroup of CFS patients with low FT3, but also controls with low T3, present a diminished responsiveness to immunologic stimuli.

Comparison with hypothyroid patients treated with T4 monotherapy

Hypothyroidism is, among others, associated with a decrease in both metabolic and heart rates, oxygen consumption, body temperature and oxidation of glucose, FA and amino acids. It has been estimated that 4–8% of genes are regulated by T3 in human skeletal muscle, rodent liver and a pituitary cell line [78]. The encountered 'low T3 syndrome' in our study resembles the thyroid hormone profile of a subgroup of hypothyroid patients receiving T4 monotherapy. Substitution with T4 is the currently recommended treatment of hypothyroid patients, like those with Hashimoto thyroiditis. Nevertheless, it is becoming increasingly clear that a subgroup of these patients experiences residual hypothyroid symptoms, including psychological and metabolic traces. These symptoms occur despite reaching a chemical euthyroid state, i.e. normal TSH [82, 83]. In thyroidectomized rats, no single dose of T4 was able to simultaneously restore TSH, T4 and T3 in plasma and organs to normal levels [84]. In so-called 'euthyroid, yet symptomatic' patients, the basal metabolic rate and serum cholesterol, among others, are not fully restored and they are also likely to have both low TT3 and FT3. These findings of low T3 may be explained by a disrupted TSH-T3 shunt [41]. The question whether they would benefit more from a T4 and T3 combination therapy or sustained-release T3 [85] is debated and subject of further research [82, 83]. Hormone replacement therapy, notably T3, has also been suggested for severe NTIS [71, 86, 87].

In the NHANES cohort, 469 out of 9,981 participants with normal TSH were T4-treated. This subgroup of T4-treated subjects exhibited 10–15% higher TT4 and FT4, but 5–10% lower TT3 and FT3 and a 15–20% lower T3/T4 ratio, as compared to 469 carefully matched healthy controls [88]. These apparently moderate differences suggest that the extrathyroidal conversion of T4 to T3 during T4 monotherapy might be insufficient in some patients to restore serum T3 levels up to those normally maintained by an intact thyroid secreting 80% T4 and 20% T3 [82, 83, 88]. A similar shift in the thyroid hormone profile was observed in the present study. However, the encountered deviations from thyroid hormone reference ranges and from controls are modest (Figure 2). It should in this context be noted that many biological effects of T3 depend on its cellular concentrations, which exhibit a complex relationship with the serum T3 concentration [89]. A recent study with chemically-induced hypothyroidism in rats showed a more severely reduced tissue T3 than serum FT3, averaging 1–6% of the baseline versus 30%, respectively. In addition, the extent of tissue T3 reduction, expressed as percentage of the baseline, was not homogeneous, with more serious reductions occurring in the order: liver = kidney > brain > heart > adipose tissue [90]. In other words, the finding of slightly decreased

circulating FT3 and perhaps also FT3 levels in the lower reference range may reflect the tip of the iceberg of the genuine T3 deficits in target tissues.

Relation with potential cause(s) of CFS

Some features of CFS resemble those of a persistent response to environmental stress known as dauer (hypometabolic state). The cell danger response (CDR) is an evolutionarily conserved metabolic response, activated when a cell encounters a chemical, physical, or microbial threat that could injure or kill the cell [91]. When the CDR is abnormally maintained, whole body metabolism and the gut microbiome become disturbed, the functionality of systems and organs becomes impaired and behavior is changed, resulting in chronic disease [91]. Accordingly, the intestinal microbiota and virome have recently been implicated in CFS [92], while gene expression data show prominent roles for genes involved in immunity and defense [93]. Psychological trauma, particularly during childhood, can also activate the CDR and produce chronic inflammation [91, 94]. It has recently been shown that CFS patients are endowed with different psychological vulnerability factors, notably perfectionism and high moral standards [95]. These may render them more susceptible to the psychological stress of current society, with possible effects on the immune system and thyroid axis [56, 62, 79, 80]. Finally, the aforementioned case-control study by Naviaux et al. [73] showed that CFS patients present cellular metabolic responses similar to the evolutionarily conserved persistent response to environmental stress. Thus, the features of hypometabolism that characterize CFS may be a consequence of a persisting CDR, either or not inflammatory driven.

The current opinion on the causes of CFS may fit mechanistically into the presently encountered 'low T3 syndrome'. We observed a shift from T3 towards rT3 in the investigated CFS patients, who exhibited lower T3/T4 ratios and higher rT3/TT3 ratios (Table 1) compared to controls. This shift towards rT3 in CFS patients was also apparent from their higher %rT3 and lower %TT3, when the sum of rT3, TT3 plus TT4 was adjusted to 100% (Table 1). These findings, as well as lower urinary iodine in CFS, may be in line with higher D3 activity. Low T3 levels in human organs have also been found in NTIS [87], but they are more likely to derive from deviant pathways of intracellular deiodination than from a seriously impaired entry of T3 into cells [87]. Induction of D3 in muscle may occur in chronic inflammation [34], but D3 may also become induced by other factors, such as estradiol, progesterone and growth hormone [96]. Such mechanisms may be at the basis of CFS symptoms and may explain the lower urinary iodine excretion in CFS patients as compared with controls, although the latter also exhibited a relatively high prevalence of low iodine excretion (Table 1). Intracellular D3-catalyzed liberation of iodide from T4 and T3 may serve various anti-oxidant and defense functions that may potentially contribute to high intracellular 'thyroid hormone consumption', manifesting as the 'low T3 syndrome' with negative iodine balance in the long term [67, 83, 97].

The lower SPINA-GD (step-up deiodinase activity) and SPINA-GT (thyroid secretory capacity) are likely to reflect thyroid allostasis responses, and the lower protein binding of thyroid hormones, as shown by the lower TT3/FT3 and TT4/FT4 ratios, may potentially result in higher metabolism/degradation of thyroid hormones. Thyroid allostasis-altered responses have been found in NTIS associated with cardiac disease [37], radiation enteritis [60] and enterocutaneous fistulas [98]. The acute adaptation of thyroid hormone metabolism to critical illness may prove beneficial to the organism, whereas the more complex alterations associated with chronic illness frequently lead to type 1 thyroid allostasis (where energy demands exceed the sum of energy intake and energy mobilized from stores) [41].

Strengths and limitations

Strength of the present case-control study is the performing of two sensitivity analyses to assess the robustness of the association of CFS with thyroid parameters and chronic (low-grade) metabolic inflammation. These analyses resulted in some differences, but the findings in thyroid parameters remained unchanged. We also calculated the %rT3, which may be a more sensitive marker for subtle metabolic shifts than concentrations and ratios *per se*.

Our study also has limitations. There was a lack of information on the duration of illness and patient characteristics at diagnosis. For instance, dependent on illness duration, different cytokine profiles in CFS patients have been reported [99]. CFS is likely a heterogeneous disease with a common final pathophysiological pathway. The present findings are possibly in line with a common final pathway, but do not get us closer to the cause(s).

Conclusion

The most remarkable finding in this CFS case-control study was a higher prevalence of the 'low T3 syndrome', attributable to a subgroup of CFS patients. Chronic low-grade metabolic inflammation was not convincingly noted. Low circulating T3 may reflect more severely depressed tissue T3 levels. The 'low T3 syndrome' might be in line with recent metabolomic studies pointing at a hypometabolic state. It also resembles a mild form of NTIS and the low T3 syndrome experienced by a subgroup of hypothyroid patients with T4 monotherapy. Our study needs confirmation and extension by others. If confirmed, trials with e.g. T3 and iodide supplements might be indicated.

ETHICS STATEMENT

All patients and controls received a verbal and written explanation of the objectives and procedures and all provided us with written informed consent. The study was in agreement with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The protocol was approved by the University Medical Center Groningen (UMCG) Medical Ethical Committee (NL44299.042.13, METc 2013/154, dated August 12, 2013).

AUTHOR CONTRIBUTIONS

BR-N, DD-B, and FM designed the research; BR-N and RT conducted the research; BR-N, RT, and EV analyzed the samples and data; BR-N, DD-B, and FM wrote the article; and FM had primary responsibility for final

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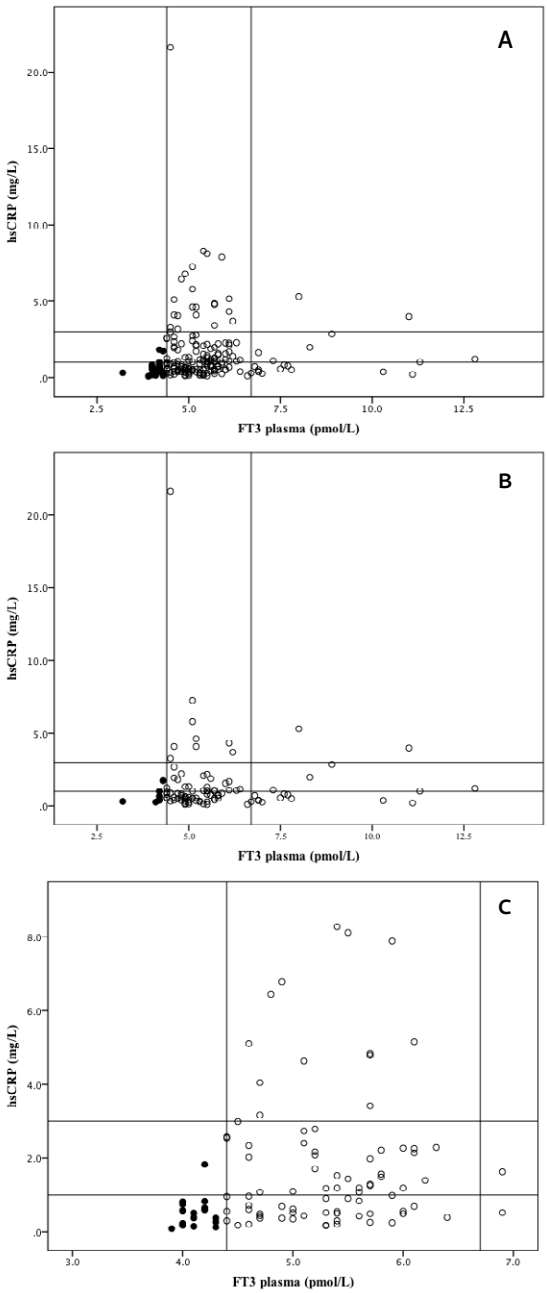
Supplementary material



Supplemental Figure 1. Relationships between FT3 and hsCRP in the whole group, controls and CFS patients.

Panel A represents the whole study group (both patients and controls); panel B, controls; and panel C, CFS patients. Vertical lines represent FT3 reference values. Horizontal lines represent hsCRP=3 mg/L and 1 mg/L.

Abbreviations: FT3, free T3; hsCRP, high-sensitive C-reactive protein.

Supplemental Table 1. Additional laboratory data of 98 CFS patients and 99 controls.

		CFS Patients	Controls			CFS Patients		Controls	
Anthropometrics	**Units**	**Median (range)**	**Median (range)**	**p value**	**Reference range/ cut–off value**	**% (n) below**	**% (n) above**	**% (n) below**	**% (n) above**
Hematology									
RBC	10^{12}/L	4.6 (3.7–5.6)	4.6 (3.8–5.8)	0.407	Men 4.6–6.2	1 (1)	0 (0)	4 (4)	0 (0)
					Women 4.2–5.4	9 (9)	0 (0)	10 (10)	2 (2)
Hemoglobin	mmol/L	8.6 (7.1–10.9)	8.5 (7.2–11.1)	0.316	Men 8.7–10.6	0 (0)	2 (2)	3 (3)	1 (1)
					Women 7.5–9.9	3 (3)	1 (1)	5 (5)	0 (0)
Hematocrit	L/L	0.40 (0.34–0.49)	0.41 (0.34– 0.51)	0.259	Men 0.42–0.52	1 (1)	0 (0)	3 (3)	0 (0)
					Women 0.37–0.47	6 (6)	0 (0)	7 (7)	0 (0)
Thrombocytes	10^9/L	261 (129–503)	252 (164–491)	0.862	150–350	1 (1)	7 (7)	0 (0)	6 (6)
Thyroid function									
Anti–TPO	IU/mL	0 (0–625)	0 (0–107)	0.355	0–35	–	5 (5)	–	4 (4)
Anti–TG	IU/mL	0 (0–2320)	0 (0–23)	0.094	0–34	–	4 (4)	–	0 (0)
Vitamins									
Folate	pmol/L	19.0 (5.5–50.0)	18.0 (8.7–50.0)	0.871	4–30	0 (0)	19 (19)	0 (0)	10 (10)
Vitamin B12	pmol/L	373 (111–1500)	284 (78–730)	<0.001**	145–450	6 (6)	41 (41)	5 (5)	11 (11)
Methylmalonic Acid	nmol/L	152.4 (67.9–371.8)	182.5 (81.9–1838.2)	0.002 **	90–340	6 (6)	3 (3)	1 (1)	7 (7)
Homocysteine	µmol/L	8.15 (3.79–37.11)	9.08 (2.57–19.03)	0.059	<10	–	30 (29)	–	31 (31)
25 (OH) Vitamin D	nmol/L	75.8 (16.0–217.2)	54.9 (5.4–133.4)	<0.001**	80–250	59 (58)	0 (0)	83 (82)	0 (0)
Minerals									
Magnesium (P)	mg/L	20.3 (14.7–24.0)	19.9 (16.5–24.3)	0.069	19.0–24.0	13 (13)	0 (0)	32 (32)	1 (1)
Zinc (P)	mg/L	0.9 (0.6–1.4)	1.0 (0.7–1.2)	<0.001**	0.8–1.4	10 (10)	0 (0)	1 (1)	0 (0)
Copper (P)	mg/L	1.1 (0.4–2.1)	1.1 (0.6–2.2)	0.560	0.7–1.4	5 (5)	18 (18)	3 (3)	15 (15)
Magnesium (IC)	mg/L	59.6 (45.8–75.4)	57.2 (40.5–81.0)	0.019*	52.0–80.0	11 (11)	1 (1)	15 (15)	0 (0)
Zinc (IC)	mg/L	15.1 (10.0–20.0)	13.7 (8.0–23.0)	<0.001**	10.5–13.7	1 (1)	79 (77)	6 (6)	48 (48)
Copper (IC)	mg/L	0.6 (0.0–0.7)	0.6 (0.0–1.0)	0.693	0.7–1.3	82 (80)	0 (0)	72 (71)	0 (0)
Zinc/Copper (IC)	g/g	25.2 (17.3–49.3)	21.8 (10.9–42.8)	<0.001**					
Zinc/Copper (P)	g/g	0.8 (0.4– 2.3)	0.9 (0.4–2.0)	0.088					
Iron (S)	µmol/L	19 (5–46)	19 (7–34)	0.889	10–30	3 (3)	5 (5)	6 (6)	4 (4)
RBC Fatty acids (g%)									
14:0	g%	0.32 (0.13–0.89)	0.35 (0.20–0.82)	<0.001**					
16:0	g%	21.14 (17.74–25.67)	22.11 (20.14–28.70)	<0.001**					
18:0	g%	17.00 (14.91–21.33)	17.19 (15.24–24.46)	0.307					
20:0	g%	0.45 (0.32–0.58)	0.45 (0.33–0.68)	0.158					
SFA	g%	45.90 (42.79–53.53)	46.25 (41.80–55.04)	0.021*					
OA	g%	12.04 (10.34–15.74)	12.29 (10.72–14.67)	0.048*					
MUFA	g%	19.36 (15.17–25.02)	18.35 (13.09–21.17)	<0.001**					
LA	g%	9.01 (6.04–12.04)	9.41 (7.23–11.73)	0.011*					
GLA	g%	0.03 (0.00–0.14)	0.04 (0.02–0.17)	0.013**					
AA	g%	14.05 (0.77–16.31)	14.09 (10.17–17.84)	0.185					
ALA	g%	0.16 (0.08–0.64)	0.17 (0.09–0.46)	0.152					
EPA	g%	0.47 (0.09–1.69)	0.53 (0.17–2.09)	0.158					
DHA	g%	3.57 (1.65–6.46)	3.52 (1.74–6.86)	0.904					
PUFA	g%	34.93 (22.40–39.92)	35.08 (25.96–43.73)	0.058					

Data are medians (ranges). Mann-Whitney U-tests were used for between-group differences.

* Significant at p < 0.05. ** Significant at p < 0.01. Abbreviations: RBC, red blood cells; anti-TPO, anti-thyroid-peroxidase antibodies; anti-TG, thyroglobulin antibodies; P, plasma; IC, intracellular; S, serum; SFA, saturated fatty acids; OA, oleic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; PUFA, polyunsaturated fatty acids

Supplemental Table 2. Anthropometrics and laboratory data of 77 CFS patients and 82 controls (selected CFS and control subgroups 2).

		CFS Patients	Controls			CFS Patients		Controls	
Anthropometrics	**Units**	**Median (range)**	**Median (range)**	**p value**	**Reference range/cut-off value**	**% (n) below**	**% (n) above**	**% (n) below**	**% (n) above**
Number		77	82						
Gender	male/ female	18/59	22/60						
Age	years	44 (21–69)	38 (19–64)	0.180					
Height	cm	172 (156–198)	173 (155–193)	0.810					
Weight	kg	65 (48–103)	67 (48–100)	0.368					
BMI	kg/m²	22 (18–30)	22 (18–29)	0.304					
Thyroid function									
TSH	mU/L	1.41 (0.62–4.40)	1.63 (0.53–3.17)	0.399	0.5–4	0 (0)	1 (1)	0 (0)	0 (0)
FT4	pmol/L	15.8 (11.4–23.0)	15.7 (11.0–19.7)	0.699	11.0–19.5	0 (0)	6 (5)	0 (0)	1 (1)
FT3	pmol/L	5.1 (3.9–6.9)	5.3 (3.2–12.8)	0.062	4.4–6.7	21 (16)	3 (2)	9 (7)	17 (14)
TT4	nmol/L	59.9 (17.8–121.3)	71.0 (45.4–134.8)	<0.001**					
TT3	nmol/L	1.3 (0.4–2.2)	1.6 (1.2 –2.3)	<0.001**					
rT3	nmol/L	0.22 (0.08–0.40)	0.23 (0.12–0.35)	0.542	0.11–0.44	1 (1)	0 (0)	0 (0)	0 (0)
% TT4		97.54 (96.69–98.44)	97.54 (96.61–98.47)	0.643					
% TT3		2.06 (1.21–2.94)	2.15 (1.24– 3.12)	0.021*					
% rT3		0.35 (0.12–1.14)	0.30 (0.15–0.45)	<0.001**					
FT3/FT4 ratio	mol/mol	0.32 (0.20–0.49)	0.34 (0.24–0.74)	0.009*					
rT3/TT3 ratio	mol/mol	0.18 (0.05–0.52)	0.15 (0.08–0.23)	<0.001**					
TT3/FT3 ratio	mol/ mmol	0.27 (0.08 –0.36)	0.31 (0.14–0.45)	<0.001**					
TT4/FT4 ratio	mol/ mmol	3.94 (1.26–6.59)	4.58 (3.15–9.05)	<0.001**					
SPINA–GT	pmol/s	1.60 (0.36–4.36)	2.01 (1.07–6.43)	0.011 *					
SPINA–GD	nmol/s	13.05 (4.36–22.94)	15.57 (10.15–25.05)	<0.001**					
sTSHi		– 1.91 (–3.08–2.51)	–1.58 (–3.21–0.70)	0.399					
Inflammation									
WBC	10^9/L	6.0 (3.3–10.0)	6.0 (3.7– 9.5)	0.160	4–10	6 (5)	–	4 (3)	–
hsCRP	mg/L	0.69 (0.09–2.74)	0.61 (0.11–2.85)	0.258	<5.0	–	–	–	–
Kynurenine	µmol/L	1.65 (0.79–2.97)	1.79 (1.05–3.03)	0.011*	1.14–3.02	12 (9)	0 (0)	2 (2)	1 (1)
Tryptophan	µmol/L	55.8 (27.9–88.7)	56.4 (43.9–90.2)	0.033*	45–83	16 (12)	1 (1)	1 (1)	1 (1)
Tryptophan/Kynurenine	mol/mol	32.53 (19.75–63.78)	32.26 (17.43–56.92)	0.622					
Ferritin[1]	µg/L	75 (8–400)	54 (5–386)	0.056	Men 30–400 Women 15–130	1 (1) 1 (1)	0 (0) 8 (6)	0 (0) 10 (8)	0 (0) 10 (8)
Urinary isoprostanes	nmol/d	1246 (164–6830)	1336 (170–9978)	0.419					
TC	mmol/L	5.2 (2.8–7.6)	5.0 (3.0–7.9)	0.970					
HDL–C[2]	mmol/L	1.5 (0.6–3.9)	1.7 (0.7–3.2)	0.009*					
LDL–C	mmol/L	3.0 (1.1–5.6)	3.1 (1.1–5.9)	0.908					
TC/HDL–C[2]	mol/mol	3.3 (1.7–10.7)	3.0 (1.7–9.0)	0.012*					
DNL Liver	g%	34.88 (32.37–39.22)	36.31 (33.58–43.85)	<0.001**					
Intestinal permeability									
Zonulin	ng/mL	1.25 (0.17–2.05)	1.36 (0.25–2.89)	0.014*					
Nutritional factors									
Urinary Iodine (24 h)	µg/d	113 (30–559)	158 (27–666)	0.001**	>200	96 (74)	3 (2)	66 (54)	23 (19)
Selenium (P)	mg/L	0.08 (0.05–0.18)	0.09 (0.06–0.13)	0.160	0.08–0.30	42 (32)	0 (0)	32 (26)	0 (0)
Selenium (IC)	mg/L	0.17 (0.11–0.63)	0.15 (0.10–0.31)	0.009*	0.17–0.55	47 (36)	1 (1)	60 (49)	0 (0)
25 (OH) Vitamin D	nmol/L	73.7 (16.0–217.2)	56.25 (5.4–133.4)	<0.001**	80–250	60 (46)	0 (0)	84 (69)	0 (0)
RBC–EPA+DHA	g%	4.03 (1.95–7.81)	4.15 (1.91–8.54)	0.448	>8	100 (77)	0 (0)	98 (80)	2 (2)
RBC EPA/AA	g%	0.03 (0.01–0.17)	0.04 (0.01–0.18)	0.220					

Data are medians (ranges). Mann-Whitney U tests were used for between-group differences. Selected subgroup 2 was created by excluding 21 CFS patients and 17 controls with hsCRP >3 mg/L and/or BMI >30 kg/m2 and/or WBC >10*109/L.

[1] When analyzed according to gender, ferritin was higher in both male and female CFS patients as compared to controls (data not shown).

[2] When the TC/HDL-C ratio and HDL-C were evaluated according to gender, we did not find differences in the TC/HDL-C ratio nor HDL-C in the relatively small number of men, but those in the females persisted (data not shown).

* Significant at $p < 0.05$. ** Significant at $p < 0.01$

Abbreviations: WBC, white blood cells; RBC, red blood cells; hsCRP, high-sensitive C-reactive protein; P, plasma; IC, intracellular; TSH, thyrotropin; FT4, free thyroxin; FT3, free triiodothyronine; TT4, total thyroxin; TT3, total triiodothyronine; rT3, reverse T3; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; SPINA-GD, sum activity of deiodinases; SPINA-GT, secretory capacity of the thyroid gland; sTSHi, standard TSH index; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DNL, de novo lipogenesis, sum of RBC 16:0,16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9, according to [61].

Supplemental Table 3. Spearman´s correlation coefficients for the relation between hsCRP and plasma TSH, FT4, FT3, TT3, TT4 and rT3 in the whole group, controls and CFS patients.

		TSH plasma (mU/L)		FT4 plasma (pmol/L)		FT3 plasma (pmol/L)		TT3 plasma (nmol/L)		TT4 plasma (nmol/L)		rT3 plasma (nmol/L)	
	Group	Correlation Coefficient	p value	Correlation Coefficient	p value	Correlation Coefficient	p value	Correlation Coefficient	p value	Correlation Coefficient	p value	Correlation Coefficient	p value
hsCRP (mg/L)	Whole group	0.043	0.548	0.054	0.453	0.173*	0.015	0.297**	<0.001	0.268**	<0.001	0.234**	0.001
	Controls	0.095	0.350	-0.029	0.775	0.078	0.446	0.338**	0.001	0.313**	0.002	0.167	0.099
	CFS patients	0.017	0.871	0.11	0.282	0.286**	0.004	0.343**	0.001	0.333**	0.001	0.309**	0.002

* Significant at p<0.05. ** Significant at p<0.01.

Abbreviations: hsCRP, high-sensitive C-reactive protein; TSH, thyrotropin; FT4, free thyroxin; FT3, free triiodothyronine; TT4, total thyroxin; TT3, total triiodothyronine; rT3, reverse T3.

Summary and Epilogue

Lifestyle and nutritional imbalances associated with Western diseases

Lifestyle, not genetics, might be the single most important primary cause of the 'typically Western' diseases of affluence. An unhealthy lifestyle is multifactorial. Strong contributors to this unhealthy lifestyle are an unbalanced diet, insufficient physical activity; poor sleep quality, chronic stress, abnormal microbial flora (microbiome) and noxious environment (e.g. smoking, fine dust). These factors exhibit interaction, but are nowadays predominantly investigated in isolation because of the reigning misconceptions of 'Evidence Based Medicine' (EBM) and 'Evidence Based Nutrition'. This first chapter aims to approach lifestyle from a holistic view, rather than from a reductionist investigation of isolated components.

Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context

With the advent of the Agricultural and Industrial revolutions, we have introduced numerous false inflammatory triggers in our lifestyle, driving us to a state of chronic systemic LGI that eventually leads to the typically Western diseases via an evolutionary conserved interaction between our immune system and metabolism. The disturbance of our inflammatory/anti-inflammatory balance is illustrated by dietary fatty acids and antioxidants. The current decrease in years without chronic disease is rather due to "nurture" than "nature," since less than 5% of the typically Western diseases are primary attributable to genetic factors.

In **Chapter 1.1** we focus on lifestyle changes, especially dietary habits, which are at the basis of chronic systemic low-grade inflammation (LGI), insulin resistance and Western diseases centered on the metabolic syndrome. The metabolic syndrome is the combination of excessive abdominal fat, impaired glucose homeostasis, hypertension and atherogenic dyslipidemia (the 'deadly quartet'). It constitutes a risk for diabetes mellitus type 2, cardiovascular disease (CVD), certain cancers, neurodegenerative diseases (e.g. Alzheimer's disease), pregnancy, fertility problems and other diseases. Systemic inflammation causes insulin resistance and compensatory hyperinsulinemia that strives to keep glucose homeostasis in balance. The goal of reduced insulin sensitivity is, among others, the reallocation of energy-rich nutrients because of an activated immune system and the repair of the inflicted damage.

Homo sapiens' sensitivity to develop insulin resistance traces back to our rapid brain growth in the past 2.5 million years. An inflammatory reaction jeopardizes the high glucose needs of our brain, causing various adaptations, including insulin resistance, functional reallocation of energy-rich nutrients and changing serum lipoprotein composition. The latter aims at redistribution of lipids, modulation of the immune reaction, and active inhibition of reverse cholesterol transport for damage repair. Resolution of the conflict between environment and our ancient genome might be the only effective manner to arrive at 'healthy aging' and to achieve this objective we might have to return to the lifestyle of the Paleolithic era according to the culture of the 21st century.

Patients undergoing elective coronary artery bypass grafting (CABG) exhibit poor pre-operative intakes of fruit, vegetables, dietary fiber, fish and vitamin D.

CVD may ensue from chronic systemic LGI. Diet is a modifiable risk factor for both and its optimization may reduce post-operative mortality, atrial fibrillation and cognitive decline. In **Chapter 1.2** we investigated the usual dietary intakes of patients undergoing elective coronary artery bypass grafting (CABG) emphasizing on food groups and nutrients with putative roles in the inflammatory/anti-inflammatory balance. Diets low in ω3-fatty acids, natural antioxidants, fiber, and vegetables and fruits, are among the pro-inflammatory factors in our diet, and the same applies for a low vitamin D status.

From November 2012 to April 2013 we approached 93 consecutive patients (80% men) undergoing elective CABG, of whom 55 were ultimately included (46 males, 9 females). For the estimation of nutritional intakes we used a food frequency questionnaire (FFQ) from the Division of Human Nutrition of Wageningen University, The Netherlands. The composed FFQ was evaluated using the mean of three 24-hour recalls as the reference method, where fair associations were encountered for vitamin D, dietary fiber, vegetables and fruit. For the same FFQ, very strong associations were found for energy intake. Patients were asked to report on their dietary habits during the previous four weeks.

Median BMI was 27 (range: 18–36) kg/m^2. Intakes (median; range) were: fruits (181; 0–433 g/day), vegetables (115; 0–303 g/day), dietary fiber (22; 9–45 g/day), eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) (0.14; 0.01–1.06 g/day), vitamin D (4.9; 1.9–11.2 µg/day), saturated fat (SFA) (13.1; 9–23 energy%) and linoleic acid (LA) (6.3; 1.9–11.3 energy%). The percentages of patients with intakes below recommendations were: 62% (fruits; recommendation: 200 g/day), 87% (vegetables; recommendation: 150–200 g/day), 73% (dietary fiber; recommendation: 30–45 g/day), 91% (EPA+DHA; recommendation: 0.45 g/day), 98% (vitamin D; recommendation: 10–20 µg/day) and 13% (LA; recommendation: 5–10 energy%). Percentages above recommendations were: 95% (SFA; recommendation: <10 energy%) and 7% (LA). Except for one patient (3% of the study population below 70 years, 2% of the total study population), none of them reported a vitamin D intake from food (median 4.9 µg/day, range 1.9–11.2) that reached the daily needs, while none of them complied with the American recommendations (for sunlight unexposed subjects: 15 µg/day for 1–69 years, and 20 µg/day for ≥70 years). Except for three patients (5%; dosage unknown), none of them reported the use of a vitamin D supplement.

We found that the dietary intakes of the patients were comparable with the average nutritional intake of the age- and sex-matched healthy Dutch population. For instance, the American Heart Association (AHA) dietary guidelines recommend the consumption of at least two servings of fish per week (particularly fatty fish), while the Dutch dietary guidelines recommend only one serving per week. At the time of the study, the recommendation was two servings of fish a week, translating to 0.45 g EPA+DHA. However, in 1998, the average fish consumption in The Netherlands amounted to hardly three times per month. The encountered unbalanced pre-operative diets may put the patients at risk of unfavorable surgical outcomes, since they promote a pro-inflammatory state. We conclude that there is an urgent need for intervention trials aiming at rapid improvement of their diets to reduce peri-operative risks. Dependent on body weight, these interventions should, in our minds, aim at iso- or hypocaloric diets, with moderate carbohydrate (CHO) (e.g. 40 energy%), moderate-high protein (e.g. 25 energy%), moderate-fat (35 energy%) and supplementation with vitamins D and B_{12}. This translates into a low-glycemic load, fiber-rich diet that is abundant in micronutrients and phytochemicals from vegetables, fruits and nuts, together with lean meat and (EPA+DHA)-rich fish.

To restore health, "do we have to go back to the future?" The impact of a 4-day Paleolithic lifestyle change on human metabolism–a pilot study.

On their way from the Stone Age via the Agricultural Revolution to current high-tech conditions, humans lost their primal foraging behavior. Today, energy expenditure is not necessary anymore for gathering nor hunting, and metabolic diseases are epidemically arising wherever our original Paleolithic lifestyle is changing towards a modern sedentary lifestyle.

In **Chapter 1.3**, we examined whether, compared to baseline, lifestyle changes towards a more Paleolithic-style pattern, correlated to changes in a variety of metabolic parameters using a within-participant design during a four-day period. In this pilot study, we followed through the concept that a radical change towards a Paleolithic hunter-gatherer lifestyle could serve as therapeutic strategy against any metaflammatory disease, even in the short term. Thirteen healthy adult volunteers were transferred to the DELUX National Park (Germany and Luxembourg) for four days and three nights, where Stone Age conditions where mimicked.

Thirty-eight biochemical and bioelectrical parameters were measured from participants before and after this relocation. There were significant decreases in body weight (-3.9%), body fat (-7.5%), body mass index (BMI) (-3.8%), visceral fat area (-14.4%) and metaflammation-related parameters (fasting glucose = -18.2%; fasting insulin = -50.1%; HOMA = -57.8%). C-reactive protein, as the main indicator for LGI, increased up to an average of 169.6 %.

Our data show that returning to our Paleolithic roots may have positive effects on risk factors commonly associated with metabolic disorders, such as obesity and diabetes mellitus type 2. The individual factors responsible for the observed benefits of our four-day immersion into the evolutionary underpinnings of diet and lifestyle are difficult, if not impossible, to allocate because of the multiple radical changes compared to a industrialized environment. Besides calorie restriction and intermittent fasting, inevitable spontaneous physical activity before food and water intake might be one of the main beneficial factors of our intervention. Another aspect is the complete disconnection from common sources

of stress associated with our modern lifestyle such as time-pressure, traffic-noise and visual complexity in exchange for old danger signals (thirst, hunger, too high or too cold temperatures) surrounded by forested landscape. Considered as natural stressors, thirst, hunger and other danger signals have accompanied us for most of our evolutionary history. It might be possible that ultimately, the synergistic effects concurring in this intervention are the main drivers responsible for such promising results.

These findings may lead the way to further research to answer the question whether the already existing metabolic conditions and/or autoimmune and neuro-inflammatory diseases could be favorably influenced by a Paleolithic lifestyle.

Influence of a 10-Day mimic of our ancient lifestyle on anthropometrics and parameters of metabolism and inflammation: the "Study of Origin".

In **Chapter 1.4** we executed another pilot study to investigate whether a 10-day mimic of a hunter-gatherer lifestyle favorably affects anthropometrics and clinical chemical indices. The absence of ancient immune challenges in current Western societies inspired us to hypothesize that acute stress from ancient danger signals causes redistribution of the immune system towards its evolutionary preferred locations and thereby favorably affects the state of chronic systemic LGI, normalizes stress axes activities, recovers rhythmic function, and restores insulin insensitive pathways. Mild stress factors may activate resolution responses based on survival mechanisms that originate from millions of years of evolutionary pressure. In this study we investigated whether such "ancient stressors," provided by a 10-day trip through the Pyrenees, improved anthropometrics and various clinical chemical parameters of LGI, stress, and metabolic control in 55 apparently healthy adults. The objective was to provide proof of principle through the notion that humans can influence their immune and metabolic systems by exposure to ancient mild acute stress factors. The intervention in our pilot study mimicked, to some extent, the "conditions of existence" of ancestral and current hunting/fishing-gathering populations.

Fifty-five apparently healthy subjects, in five groups, engaged in a 10-day trip through the Pyrenees. They walked 14 km/day on average, carrying an eight-kilo backpack. Raw food was provided and self-prepared and water was obtained from waterholes. They slept outside in sleeping bags and were exposed to temperatures ranging from 12 to 42°C. Anthropometric data and fasting blood samples were collected at baseline and the study end.

We found important changes in most outcomes favoring improved anthropometrics and enhanced metabolic function. Body weight decreased with a median (range) of −3.8 kg (−12.5 to −0.7), BMI with −1.2 kg/m^2 (−4.4 to −0.2), hip circumference with −3 cm (−17 to +5), waist circumference with −5 cm (−18 to +9) and waist/hip ratio with −0.02 (−0.14 to +0.10). We also observed decreases (median; range) in: glucose (−0.6; −1.7 to +0.5 mmol/L), HbA1c (−0.1; −0,4 to +0.2 %), insulin (−4.7; −31.4 to −0.2 pmol/L), HOMA-IR (−1.2; −7.0 to −0.4 mmol*mU/L^2), triglycerides (−0.14; −6.12 to +2.18 mmol/L), total cholesterol (TC) (−0.7; −2.8 to +0.4 mmol/L), LDL-cholesterol (LDL-C) (−0.6; −3.1 to +0.6 mmol/L), triglycerides/HDL-cholesterol (HDL-C) ratio (−0.55; −8.98 to 1.34 mol/mol), and FT3 (−0.8; −3.4 to +3.1 pmol/L). On the other hand, we found that ASAT and ALAT activities increased with 11 IU/L (−8 to 54) and 6 IU/L (−13 to 52), respectively, while C-reactive protein (CRP) increased with 0.56 mg/L (−15.72 to +41.07). The ASAT/ALAT ratio increased with 0.08 to 1.31 (0.48–2.06).

Our interventions might be based on causing 'mild acute stress' in humans who in their usual daily lives are exposed to chronic stress, commensurate with our modern lifestyle. Acute stress promotes release of stress hormones, including adrenaline, noradrenaline and cortisol, that each cause profound metabolic and immunologic adaptations. Acute stress factors increase autonomic activity, accelerate immune cell proliferation and differentiation, and also stimulate the anti-inflammatory component of the immune system (i.e. production of IL10, lactoferrin, lysozyme). Nevertheless, mild stress initially produces a pro-inflammatory response, which may subsequently give rise to recovery from the reigning state of chronic LGI and the return to homeostasis.

A short period of return to the 'conditions of existence' similar to those on which our genome is based may improve anthropometrics and metabolism by favorably challenging the immune system in apparently healthy subjects and possibly patients with fibromyalgia. The 'return' may come with some costs in more active infection, as a trade-off for the chronic systemic LGI typical of our current lifestyle of affluence. We may increasingly appreciate that we cannot have

it all, while the evolutionary lessons of Darwin and intervention studies teach us that prevention might be more rewarding and affordable than the current culture of medical treatment.

Saturated fatty acids (SFA)

There is much controversy about the influence of SFA on cardiovascular disease (CVD). The reigning contention is that dietary SFA are detrimental and that their intakes, notably those of palmitic- (16:0), lauric- (12:0) and myristic- (14:0) acids, should be limited or be 'as low as possible'. Palmitic acid is abundant in e.g. animal fat (human milk included) and palm oil, while both lauric- and myristic acids are abundant in coconut oil, but also in human milk.

The relation of saturated fatty acids with low-grade inflammation and cardiovascular disease.

The mantra that dietary (saturated) fat must be minimized to reduce CVD risk has dominated nutritional guidelines for decades. In view of current controversies regarding their adequate intakes and effects, **Chapter 2.1** aims to summarize research regarding this heterogenic group of fatty acids and the mechanisms relating them to (chronic) systemic LGI, insulin resistance, metabolic syndrome and notably CVD.

The interest in the relation between dietary fat and CVD arose from animal studies indicating that dietary cholesterol caused arterial lesions, largely mediated through an elevation of blood cholesterol levels. Since then, the relation between dietary fat and CVD risk has been intensively studied, using different approaches, including controlled feeding studies, RCTs and large cohort studies. Most of the studies on SFA solely focused on their tendency to alter lipoprotein metabolism and to influence the concentrations of lipoproteins carrying cholesterol, among other lipids, in blood. From these studies, the question of what constitutes the healthiest overall mixture of the different classes of dietary fats remains unanswered. The indisputably high human milk SFA content is testimony of their beneficial effects, at least in breastfed infants for, among others, their antimicrobial properties and as rapidly available energy sources.

The intimate relationship between inflammation and metabolism, including the metabolism of glucose, fat and cholesterol, revealed that the dyslipidemia in Western societies, notably increased triglycerides, "small dense" low-density lipoprotein and "dysfunctional" high-density lipoprotein, is influenced by many unfavorable lifestyle factors. As Paracelsus (1493–1541) already stated, 'It is the dose (and circumstances) that makes the poison'. Our food is composed of complex biological systems, such as meat, fish, vegetables and fruits, in which the nutrients, SFA included, obey to the balance that comes along with living material. It is this balance on which hominins have evolved that may at best support our health. Moreover, it is important to gain insight into the interaction of the various lifestyle factors. Dietary SFA is only one of the many lifestyle factors playing a role in chronic systemic LGI and the subsequent metabolic adaptations, including those causing changes in the concentrations of circulating lipids and lipoprotein-cholesterol. The environment provides us with many other pro-inflammatory stimuli than SFA, but also with many compensating anti-inflammatory stimuli. It is all about 'balance'.

We content that it is the interaction between many lifestyle factors that determines whether SFA, and as a matter of fact any nutrient, contributes to systemic LGI, changes in lipoprotein metabolism and ultimately CVD risk. The dysbalance between proinflammatory and anti-inflammatory stimuli in our Western society does not originate from a single cause and, consequently, may also not become solved by a single 'magic bullet'. Resolution of the conflict between our self-made environment and our ancient genome may rather rely on returning to the lifestyle of the Paleolithic era according to the culture of the 21st century. Accordingly, dietary guidelines might reconsider recommendations for replacing SFA, since 'food, not nutrients, is the fundamental unit in nutrition'. Diet should be investigated in a broader context, together with non-dietary lifestyle factors. This should be a clear priority, as opposed to the reductionist approach of studying the effects of single nutrients, such as SFA.

2.1.a Notes added to the SFA review in August 2017

In **Chapter 2.1.a**, based on the recent 'White paper' of Pett et al., it has come to our attention that we have mistakenly attributed Figure 1 in **Chapter 2.1** to The Seven Countries Study originated by Ancel Keys. This Figure derives from the paper of Yerushalmy and Hilleboe, published one year prior to the initiation of The Seven Countries Study. This paper and its figures have been used to illustrate the findings of The Seven

Countries Study in the literature and social media, but they actually do not. Based on these data, Keys has been falsely accused of 'cherry picking' to make his point of a relation between SFA intake and CVD. Our Figures 1A and B were not produced in the same study, and neither of them was from the Seven Countries Study. Given the inherently unreliable nature of food data and mortality prior to The Seven Countries Study, the question still remains of what were the criteria used by Keys for the selection of those six countries represented on the Figure from 1953.

Despite our above-mentioned omission, we do not change our opinion regarding the influence of dietary SFA on CVD. The 'White Paper', however, did not aim to target this issue, explaining that it 'does not espouse or promote any dietary advice; it is intended only to present a historically accurate account of well-documented work and redress misrepresentations of that work'. The latter refers to the work of Keys as the originator of The Seven Countries Study.

Saturated fatty acid (SFA)-status and SFA-intake exhibit different relations with serum total cholesterol and lipoprotein-cholesterol: a mechanistic explanation centered around lifestyle-induced low grade inflammation.

Total cholesterol/HDL cholesterol ratio is a widely used CVD risk factor. In **Chapter 2.2** we investigated the relations between fatty acid-status and serum TC, LDL-C, HDL-C and TC/HDL-C ratio in five Tanzanian ethnic groups and one Dutch group, and studied whether these correlations are different from the reported effects of SFA-intake in Western societies. The studied ethnic groups differ widely in the intakes of both SFA and LA. Fatty acid status was determined by measurement of fatty acids in serum cholesterol esters and erythrocytes (RBC). Data reflecting the influence of fatty acid intakes on serum total cholesterol and lipoprotein cholesterol were obtained from documented intervention studies.

The inhabitants of the island of Chole have high intakes of local marine fish and coconut and consume plenty amounts of free fruit and vegetables. They do not use vegetable oils for cooking and have low intakes of CHO from grains or corn. Both the Maasai Ruvu and Wasso diets consist of curdled milk andmeat from their own stock, which has recently become replenished with *ugali* (maize porridge). Whole carcass meat consumption is a regular practice among the Maasai. Fish is usually not eaten, while vegetables and fruits are considered foods for cows. The people from Sengerema have a regular fish intake (average 4–5 times/week). Ugali, muhogo (cassava root) and plantain (baked banana) are staple foods. The Hadzabe are traditional hunter–gatherers whose diet is composed of berries, roots, honey, meat and an occasional fish. They hunt small animals in the wet season and bigger game in the dry season. In recent years, corn and corn oil have inevitably become a large part of their contemporary diet. Dietary fat intake in the Netherlands comprises about 34 en%, and is, after CHO, the main energy source. Average SFA, monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) intakes are 12.5, 12.7 and 6.3 en%, respectively. Dairy products, meat and meat products, fat and cakes are the main sources of dietary fat and SFA in the Netherlands. Grains, grain products and non-alcoholic beverages are the most important sources of dietary CHO. More than 75% of the current adult Dutch population does not adhere to the recommendation of consumption of 200 g fruit and 200 g vegetables per day, nor the consumption of one fish serving per week (particularly fatty fish) in adults. In fact, the average fish consumption hardly amounts to three times per month, the intake of salt is regarded as much too high and so is the intake of CHO with high glycemic indices.

We found that 14:0-, 16:0- and SFA-status, but **not** their intakes, correlate positively with the TC/HDL-C ratio. LA- and PUFA-status and PUFA-intake exhibited negative relations with the TC/HDL-C ratio. Focusing on the TC/HDLC ratio, the often used CVD risk factor, we found that: 1) based on FA-status, 14:0, 16:0 and SFA have detrimental effects, while the 14:0-, 16:0- and SFA-intakes are neutral; 2) that both 18:0-status and 18:0-intake are neutral; 3) that the MUFA-status is neutral while the MUFA-intake is beneficial; 4) that the LA- and PUFA-status are beneficial and so is the PUFA-intake; and finally, 5) that the EPA-status is detrimental.

Our data suggest that, taken from the TC/HDLC ratio, a high SFA-status, **not** a high SFA intake, is associated with increased CVD risk, while both high LA-status and PUFA-status are associated with reduced CVD risk. Consequently, the TC/HDL-C ratio is a questionable risk marker, since meta-analyses of RCTs show that partial dietary replacement of SFA for LA, the dominating dietary PUFA, does not change CVD risk. This replacement has been shown to cause

a borderline insignificant higher death from CVD (hazard ratio 1.33; 95% confidence limit 0.99–1.79). We conclude that many lifestyle factors, not SFA-intake alone, determine SFA-status, and suggest that interaction with many other lifestyle factors determines whether SFA-status has a relevant contributing effect in LGI, lipoprotein changes and CVD risk. The present outcome may teach us to consider the health effects of the entire diet together with many non-dietary lifestyle factors, opposite to the reductionist approach of studying the effects of single nutrients, SFA and PUFA included.

Comment on the report ´Dietary Fats and Cardiovascular Disease: A Presidential Advisory from the American Heart Association (AHA)´.

Recently, the American Heart Association (AHA) published a meta-analysis emphasizing their earlier recommendation to limit the intake SFA. SFA should be replaced with unsaturated fat, especially PUFA, to lower the incidence of heart disease. Such replacement is claimed to reduce the risk for cardiovascular events by about 30%; a risk reduction comparable to treatment with statins. The AHA also advises against coconut oil consumption because it increases LDL-C and ´has no known offsetting favorable effects'. We argue that the LDL-C concentration is still a soft endpoint, not a disease, while there are no studies showing unfavorable effects of coconut oil on hard endpoints. The AHA extensively motivates the exclusion of studies for their meta-analysis, but does not apply stringent criteria in the choice of the four trials constituting the backbone of their final meta-analysis. One of these was not a randomized controlled trial, while another suffered from 'performance bias'. The largest negative trial was excluded, amongst others, because it did not last at least two years. The AHA meta-analysis conveys the notion of 'cherry picking'. There are at present at least nine expert reviews that failed to find a clear link between SFA, cardiovascular mortality and total mortality. We argue that individuals with the metabolic syndrome should be careful with dietary SFA and carbohydrate, since they synthetize SFA *de novo* from carbohydrates and spare dietary SFA. The high risk of individuals with the metabolic syndrome is no reason to limit SFA intake of the genuinely healthy population. Some SFA are definitely pro-inflammatory, but a balanced diet also contains anti-inflammatory components.

Astaxanthin, the pink carotenoid

Chapter 3 focuses on astaxanthin. Astaxanthin is a unique carotenoid of predominantly marine origin. The natural form functions as an antioxidant without pro-oxidant properties or side effects after oral intake. Astaxanthin belongs to the xanthophyll family, providing the pink-red color to certain microalgae (i.e. *Haematococcus pluvialis*) and accumulates in various animals higher in the food chain such as flamingoes, salmon, shrimps and crayfish. It is likely that astaxanthin is part of the land-water ecosystem from which *Homo sapiens* derives. The molecule spans the phospholipid double layer of cell membranes due to its two polar head groups that are interspaced by a branched carbon atom chain containing nine conjugated double bonds. Astaxanthin has been found to enhance the immune response, to decrease oxidative damage-related symptoms and has been shown effective in several diseases and conditions, such as Alzheimer's disease, obesity, asthma, enlarged prostate, osteoarthritis and rheumatoid arthritis.

Kinetics of plasma- and erythrocyte-astaxanthin in healthy subjects following a single and maintenance oral dose.

Chapter 3.1 presents the kinetics of astaxanthin in healthy subjects. Following its ingestion, astaxanthin has been shown to reach a peak in plasma at about 7 h and to decline with a median half-life of about 21 h. We investigated astaxanthin kinetics in plasma and RBC of four healthy adults after a single oral 40 mg dose. Plasma- and RBC-astaxanthin were measured during 72 h. Subsequently, an 8 mg/day dose was given during 17 days. Plasma- and RBC-astaxanthin were measured each morning.

Plasma-astaxanthin reached a peak (from 79 to 315 nmol/L) after 8 h and then declined (half-life, 18 h). Within 72 h, plasma-astaxanthin had returned to baseline. RBC-astaxanthin reached a peak (from 63 to 137 nmol/L packed cells) at 12 h and subsequently disappeared (half-life, 28 h). During the daily 17-day dose, plasma-astaxanthin increased until day 10 (187 nmol/L) and then decreased to a steady concentration similar to that reached after 2 days. RBC-astaxanthin appeared to be highly variable (group median concentration, 86 nmol/L packed cells).

We found high intra- and inter-individual variations, especially in RBC, possibly due to non-standardized time difference between astaxanthin intake

and sampling, fluctuating background intake from the diet, variable bioavailability, large distribution volume, degradation or others. Oral astaxanthin is rapidly absorbed and incorporated into RBC. The short plasma- and RBC-astaxanthin half-lives of 18 and 28 h, respectively, suggest the necessity to take astaxanthin on a daily basis to maintain a higher-than-baseline steady state, at least in the initial phase of supplementation when total body equilibrium, if any, has not been reached yet. This early phase might in part be influenced by the tendency of astaxanthin to incorporate into all bodily cell membranes.

Supplementation of patients with sickle cell disease with astaxanthin increases plasma- and erythrocyte-astaxanthin and may improve the hemolytic component of the disease.

Sickle cell disease (SCD) is a heterogeneous disorder that is mechanistically characterized by hemolytic and vaso-occlusive components. The latter gives rise to cumulative ischemic organ damage that may occasionally precipitate to painful vaso-occlusive crises; all jointly contributing to diminished quality of life and early death. The hemolytic component may find an important trigger in the generation of ROS by sickle hemoglobin (HbS) close to the lipid peroxidation-sensitive RBC membrane, ending up in hemolysis if the challenge is not appropriately opposed. The vaso-occlusive component may be largely driven by the aforementioned hemolytic component. Targeting the hemolytic component, and notably oxidative stress, by amelioration of the devastating vaso-occlusive component, seems a logical intervention strategy. Various trials with naturally occurring antioxidants with promising outcomes have been reported, including those with vitamin E, curcuminoids, aged garlic extract, N-acetylcysteine and zinc. Due to its unique antioxidant properties, astaxanthin supplementation might ameliorate the hemolytic component of SCD.

Chapter 3.2 presents an open label pilot intervention study, where we included 10 laboratory-confirmed SCD outpatients (seven adults, three children, of which three males and seven females, mean age 31 years, range 6–52 years) from the Sint Maarten Medical Centre. We investigated the effect of a daily 8–12 mg oral dose during 3 months, on plasma- and RBC-astaxanthin levels (primary goal) and several hematological and clinical chemical parameters (secondary goal), including reticulocyte count, mean corpuscular volume (MCV), RBC distribution width (RDW), lactate dehydrogenase (LDH) and asymmetric dimethylarginine (ADMA).

Baseline plasma (33 nmol/L) and RBC (11 nmol/L packed RBC) astaxanthin increased to 225, 174, 167 nmol/L (plasma) and 149, 100, 71 nmol/L packed RBC at 1, 2 and 3 months, respectively. Reticulocytes decreased from baseline and 2 months (9.5 and 8.8%) to 3 months (5.6%), MCV from 2 to 3 months (88 to 86 fL), MCH from baseline to 3 months (30 to 28 pg) and RDW from baseline and 2 months (19.2 and 19.0%) to 3 months (16.7%). Plasma arginine decreased from 2 to 3 months (46.6 to 39.4 µmol/L). Astaxanthin supplementation did not change homoarginine, ADMA, symmetric dimethylarginine (SDMA) and the ADMA/arginine ratio. Reticulocytes at baseline correlated with relative changes in reticulocytes from baseline to 3 months. Relative changes in reticulocytes correlated with relative changes in RBC, RDW, LDH, ALAT, but not hematocrit, within the same period.

We concluded that astaxanthin incorporates into SCD RBC and may favorably affect the hemolytic component. We found a slight reduction of the reticulocyte count after 3 months, probably indicating lower hemolysis; while many of the patients in this non-placebo-controlled, non-randomized, trial reported that they 'felt better'. A larger RCT is indicated, using similar or higher dose, preferably during more than 3 months. It might be even better to include astaxanthin into a supplemental mix with other antioxidants (e.g. low-dose vitamin E, beta-carotene, vitamin C and folic acid), minerals (selenium if necessary; and notably zinc;), amino acids (notably arginine), fish oil and vitamin D. Antioxidants do not work on their own but are rather part of a yet poorly understood antioxidant network of free radical scavengers, quenchers and antioxidant enzymes and therefore, it seems improbable to find a single "magic bullet" to prevent or treat any disease associated with oxidative stress.

Higher prevalence of 'low T3 syndrome' in patients with chronic fatigue syndrome: A case-control study.

Chronic fatigue syndrome (CFS) is a heterogeneous disease with unknown cause(s). Many pathophysiological cascades have been hypothesized but underlying organic conditions are rarely found. Disturbed hypothalamus-pituitary-adrenal (HPA) axis, presented as

mild hypocortisolism, heightened negative feedback and blunted responses to challenge have been found in CFS. CFS symptoms resemble a hypothyroid state, possibly secondary to chronic (low-grade) (metabolic) inflammation. We might be dealing with the human equivalent of 'hibernation' with causes rooted in a typically Western lifestyle that triggers LGI.

In **Chapter 4**, we present a CFS case-control study. We investigated 98 CFS patients (21–69 years, 21 males) and 99 age- and sex-matched controls (19–65 years, 23 males). We measured parameters of thyroid function, (metabolic) inflammation, gut wall integrity and nutrients influencing thyroid function and/or inflammation.

Most remarkably, CFS patients exhibited similar TSH, but lower free T3 (FT3) (difference of medians 0.1%), total T4 (TT4) (11.9%), total T3 (TT3) (12.5%) and %TT3 (4.7%), higher % reverse T3 (rT3) (13.3%), and lower 24-h urinary iodine (27.6%). FT3 below the reference range, consistent with the 'low T3 syndrome', was found in 16/98 CFS patients vs. 7/99 controls (OR 2.56; 95% CI=1.00–6.54). Most observations persisted in two sensitivity analyses with more stringent cut-off values for BMI, hsCRP and WBC. We found possible evidence of (chronic) low-grade metabolic inflammation (ferritin and HDL-C). FT3, TT3, TT4 and rT3 correlated positively with hsCRP in CFS patients and all subjects. Only TT3 and TT4 correlated positively with high-sensitive CRP (hsCRP) in controls.

Low circulating T3 and the apparent shift from T3 to rT3 may reflect more severely depressed tissue T3 levels. The present findings might be in line with recent metabolomic studies pointing at a hypometabolic state. They resemble a mild form of 'non thyroidal illness syndrome' and the 'low T3 syndrome' experienced by a subgroup of hypothyroid patients receiving T4 monotherapy. Our study needs confirmation and extension by others. If confirmed, trials with e.g. T3 and iodide supplements might be indicated. CFS is likely a heterogeneous disease with a common final pathophysiological pathway. The present findings are possibly in line with a common final pathway, but do not get us closer to the cause(s).

Epilogue. Applicability of an evolutionary approach to (chronic) diseases and prevention

'Evolutionary Medicine' is gaining acceptance. The influential journal 'The Lancet" recently published three papers and an editorial on the subject [1–4]. Logic predicted, but now also science shows, that Lifestyle, not Genetics, is the single most important primary cause of the 'typically Western' diseases of affluence [5].

In this thesis, we hope to have contributed to the concept above, and more specifically, to the awareness that 'healthy aging' is a lifestyle matter. The components of an unhealthy lifestyle are unbalanced diet, insufficient physical activity, inadequate sleep, chronic stress, abnormal microbial flora (microbiome), and noxious environment (e.g. smoking, fine dust). Because of their interaction, it may not be so productive to merely study these risk factors in isolation, whether by means of RCTs or not.

There has been, and still is, little attention for the limitations of RCTs and certainly not for the limitations of the meta-analyses thereof. Rawlins [6] noted that: "hierarchies of evidence should be replaced by accepting—indeed embracing—a diversity of approaches". Nevertheless, RCTs and their meta-analyses are widely regarded to reflect the 'highest level of evidence', and by some even 'the only ones to consider in making guidelines'. This approach, most appropriate for drugs, but not necessarily for nutrients, has reached the current status of a 'paradigm', defined as 'a shared understanding among scientists or scholars working in a discipline regarding the important problems, structures, values and assumptions determining that discipline' [7].

From the beginning, RCTs and their meta-analyses were not meant to constitute the only components of 'Evidence Based Medicine/Nutrition', at least not in the minds of the original inventors [8, 9]. Moreover, current meta-analyses often arrive at opposing conclusions, even after reading exactly the same literature. An example is the recent meta-analysis-based recommendation for saturated fat issued by the AHA, which contradicts 'a total of at least 17 meta-analyses and systematic reviews and five non-systematic reviews that have failed to find a clear link between saturated fats and heart disease/cardiovascular death' [10]. Selective omission of studies because of 'poor quality', possibly inspired by biased opinion, also named 'cherry picking', might have become the new trend, and is at least in the center of the discussion nowadays [11–13].

Such controversies do not confer great confidence in science and frustrates the public. Nowadays, both learned non-insiders and laypeople have virtually unlimited access to scientific literature, whereas any

'scientific opinion' should not be taken for granted, even deriving from e.g. an authoritative scientist (also named 'Eminence Based Medicine'), Health Council or Government.

What nowadays (most of the) public and (most of the) Governments understand is the concept of 'sustainability', defined in ecology as 'the property of biological systems to remain diverse and productive indefinitely' [14]. Now that the consequences of e.g. global warming, are starting to manifest, piercing questions are increasingly arising, including 'what is in my food', 'how is it produced', and 'why does not my grandmother recognize this as edible'.

The public understands, possibly even better than many scientists, that we have created a conflict between our environment and our ancient genome. The latter cannot adapt, neither genetically (long term), nor epigenetically (shorter term), to environmental challenges occurring at a still increasing pace. The controversy is not only due to the nowadays frequently blamed "bloggers" [15–18], and not only about saturated fat (as one of the subjects in this thesis) but also CHO, LA, salt and vitamin D, among others.

In view of the above, we conclude that the only effective manner to arrive at 'healthy aging' might be returning to the lifestyle of the Paleolithic era according to the culture of the 21st century. And that is exactly what 'Evolutionary Medicine' is about.

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Samenvatting en Epiloog

Levensstijl en ongebalanceerde voeding zijn geassocieerd met westerse ziekten

Levensstijl is, in plaats van genetica, mogelijk de belangrijkste oorzaak van de ´typisch westerse´ welvaart ziekten. Een ongezonde levensstijl is afhankelijk van meerdere factoren, met als belangrijke bijdragers een onevenwichtig voedingspatroon, onvoldoende lichamelijke activiteit; slechte slaapkwaliteit, chronische stress, abnormale microbiële flora en een schadelijke omgeving (bijvoorbeeld roken of fijnstof). Deze factoren interacteren met elkaar, terwijl ze overwegend in isolatie worden onderzocht, dit door de heersende misvattingen van '*Evidence Based Medicine*' en '*Evidence Based Nutrition*'. Dit eerste hoofdstuk benadert daarom levensstijl juist vanuit een holistisch oogpunt, in plaats van een reductionistisch onderzoek naar de verschillende geïsoleerde componenten.

Levensstijl en ongebalanceerde voeding zijn geassocieerd met westerse ziekten: oorzaken en gevolgen van chronische systemische lage mate van ontsteking in een evolutionaire context

Met de komst van de landbouw- en industriële revoluties hebben we in onze levensstijl talloze onterechte ontstekingsveroorzakers geïntroduceerd, welke leiden tot een toestand van chronische systemische ontsteking, die uiteindelijk leidt tot de typische westerse ziekten, via een evolutionair geconserveerde interactie tussen ons immuunsysteem en metabolisme. De onderliggende oorzaken zijn een abnormale dieet samenstelling in combinatie met microbiële flora, onvoldoende fysieke activiteit en slaap, chronische stress en milieuvervuiling. De verstoring van onze ontstekingsbalans wordt geïllustreerd door voedingszuren en antioxidanten. De huidige daling van het aantal jaren zonder chronische ziekte is eerder te danken aan opvoeding dan aanleg, omdat minder dan 5% van de typisch westerse ziekten primair toe te schrijven zijn aan genetische factoren.

In **Hoofdstuk 1.1** richten wij ons op levensstijlveranderingen, met name voedingspatronen die aan de basis liggen van chronische systemische lage graad ontsteking (LGI), insulineresistentie en westerse ziekten die geassocieerd zijn met het metabolische syndroom. Het metabolische syndroom is de combinatie van overmatig buikvet, verminderde glucose homeostase, hypertensie en atherogene dyslipidemie (het 'dodelijke kwartet'). Het verhoogt het risico op diabetes mellitus type 2, hart- en vaatziekten (CVD), bepaalde typen kanker, neurodegeneratieve ziekten (bijvoorbeeld de ziekte van Alzheimer) en andere aandoeningen. Systemische ontsteking veroorzaakt insulineresistentie en als gevolg daarvan hyperinsulinemie om de glucose homeostase in evenwicht te houden. Het doel van deze verminderde insuline gevoeligheid is onder andere de herverdeling van energierijke voedingsstoffen door een geactiveerd immuunsysteem en het herstel van de toegediende schade.

De gevoeligheid van *homo sapiens* voor het ontwikkelen van insulineresistentie is te herleiden naar onze snelle hersengroei over de laatste 2,5 miljoen jaar. Een ontstekingsreactie brengt namelijk de hoge glucosebehoeften van onze hersenen in gevaar, daarom vinden verschillende aanpassingen plaats, waaronder insulineresistentie, functionele herverdeling van energierijke voedingsstoffen en het veranderen van de serum lipoproteïne samenstelling. Dit laatste richt zich op herverdeling van lipiden, modulatie van de immuunreactie en actieve remming van omgekeerd cholesterolvervoer voor schadeherstel. Voor *´healthy aging´* is er een oplossing nodig voor het conflict tussen milieu en ons oud genoom. Een mogelijk antwoord licht in het terugkeren naar de levensstijl van het Paleolithische tijdperk volgens de cultuur van de 21ste eeuw.

Patiënten die coronary artery bypass grafting (CABG) ondergaan, hebben een slechte preoperatieve inname van fruit, groenten, voedingsvezels, vis en vitamine D

Chronische systemische LGI kan CVD tot gevolg hebben. Voor zowel LGI als CVD is dieet een risicofactor en een geoptimaliseerd voedingspatroon kan de kans op post-operatief overlijden, hartritmestoornissen en cognitieve achteruitgang verkleinen. In hoofdstuk 1.2 onderzoeken we het dagelijkse voedingspatroon van patiënten die *coronary artery bypass grafting* (CABG) ondergaan. Hierbij leggen we de nadruk op voedselgroepen en nutriënten die een belangrijke rol spelen in de ontstekingsbalans. Factoren die ontstekingsbevorderend werken zijn het eten van weinig ω3-vetzuren, natuurlijke antioxidanten, vezels, groente en fruit. Ook het hebben van een laag vitamine D gehalte behoort hiertoe.

Tussen November 2012 en april 2013 hebben we 93 CABG patiënten benaderd (80% man), waarvan er uiteindelijk 55 zijn geïncludeerd (46 mannen, 9 vrouwen). Om inzicht te krijgen in het dagelijks

voedingspatroon hebben we de '*food frequency questionaire*' (FFQ) van de Division of Human Nutrition van Universiteit Wageningen gebruikt. De resultaten van de FFQ werden geëvalueerd door het gemiddelde van drie 24 uurs beschrijvingen als referentie te nemen. Relaties werden gevonden tussen vitamine D, voedingsvezels, groente en fruit. Tevens vond de FFQ een sterke associatie met energy opname. Patiënten werden vervolgens gevraagd om hun voedingspatroon over de voorgaande vier weken te rapporteren.

De mediaan BMI was 27 kg/m^2 (bereik: 18–36). Voedingsinname (mediaan; bereik) was: fruit (181; 0–433 g/dag), groenten (115; 0–303 g/dag), voedingsvezel (22; 9–45 g/dag), eicosapentaeenzuur (EPA) + docosahexaeenzuur (DHA) (0,14; 0,01–1,06 g/dag), vitamine D (4,9; 1,9–11,2 µg/dag), verzadigd vet (SFA) (13,1; 9–23 energie%) en linolzuur (LA) (6,3; 1,9–11,3 energie%). Het percentage patiënten met inname onder de aanbevelingen waren: 62% fruit (aanbeveling: 200 g/dag), 87% groenten (aanbeveling: 150–200 g/dag), 73% voedingsvezel (aanbeveling: 30–45 g/dag), 91% (EPA + DHA (aanbeveling: 0,45 g/dag), 98% vitamine D (aanbeveling: 10–20 µg/dag) en 13% LA (aanbeveling: 5–10 energie%). De percentages boven de aanbevelingen waren: 95% SFA (aanbeveling: <10 energie%) en 7% (LA). Behalve één patiënt (3% van de onderzoekspopulatie onder 70 jaar, 2% van de totale studiepopulatie) meldde geen van hen een vitamine D-inname uit voedsel die toereikend was voor de dagelijkse behoefte (mediaan 4,9 µg/dag, bereik 1,9–11,2), terwijl geen van hen voldoet aan de Amerikaanse aanbevelingen voor blootstelling aan zonlicht (15 µg/dag voor 1–69 jaar en 20 µg/dag gedurende 70 jaar). Behalve voor drie patiënten (5%, onbekend dosis), meldde geen van hen het gebruik van een vitamine D-supplement.

We hebben gevonden dat de voedingsinname van de patiënten vergelijkbaar was met de leeftijd- en geslacht gekoppelde gezonde Nederlandse bevolking. De voedingsrichtlijn van de American Heart Association (AHA) raad de consumptie aan van minstens twee porties vis per week (vooral vetvis), terwijl de Nederlandse voedingsrichtlijnen slechts één portie per week aanbevelen. Op het moment van de studie was de aanbeveling twee porties vis per week, wat in onze studie is vertaald naar 0,45 g EPA + DHA.

In 1998 bedroeg het gemiddelde visverbruik in Nederland echter nauwelijks drie keer per maand. Het geconstateerde onevenwichtige pre-operatieve dieet kan de patiënten in gevaar brengen voor ongunstige chirurgische uitkomsten, aangezien deze een pro-inflammatoire toestand kan bevorderen. We concluderen dat er dringend behoefte bestaat aan interventies die gericht zijn op een snelle verbetering van de juiste voedingsinname om pre-operatieve risico's te verminderen. Afhankelijk van het lichaamsgewicht moeten deze interventies op iso- of hypocalorische diëten gericht zijn, met matige koolhydraten (CHO) (bijvoorbeeld 40 energie%), matig hoog eiwit (bijv. 25 energie%), matig vet (35 energie%) en toevoeging van vitaminen D en B12. Dit vertaalt zich in een vezelrijk dieet met laagglycemische lading met veel micronutriënten en fytochemicals uit groenten, fruit en noten, samen met mager vlees en (EPA + DHA) -rijke vis.

Moeten we terug naar de toekomst om gezondheid te herstellen? De impact van een 4-daagse Paleolithische levensstijlverandering op menselijk metabolisme - een proefonderzoek

Op de weg van de Steentijd via de Landbouwrevolutie naar de huidige hightech-omstandigheden, verloor de mens zijn primale voedingsgedrag. Vandaag zijn energie uitgaven niet meer nodig om te verzamelen of te jagen, en metabolische ziekten komen als een epidemie op, daar waar onze oorspronkelijke Paleolithische levensstijl verandert in een moderne, sedentaire levensstijl.

In **Hoofdstuk 1.3** hebben we onderzocht of, veranderingen richting een meer Paleolithische levensstijl, gecorreleerd zijn met veranderingen in een verscheidenheid aan metabolische parameters met behulp van een binnen-deelnemers onderzoeksopzet, vergeleken met hun normale voedingspatroon gedurende een periode van vier dagen. In dit proefonderzoek hebben we getest of een radicale verandering in de leefstijl richting een Paleolithische jager-verzamelaar op korte termijn als therapeutische strategie zou kunnen dienen tegen ontstekingscomplicaties. Dertien gezonde volwassen vrijwilligers werden voor vier dagen en drie nachten overgebracht naar het DELUX National Park (Duitsland en Luxemburg), waar de levensomstandigheden van het stenen tijdperk werden nagebootst.

Drieëndertig biochemische en bio-elektrische uitkomstmaten werden gemeten van deelnemers vóór en na deze verhuizing. Er waren significante afname van het lichaamsgewicht (–3,9%), lichaamsvet (–7,5%), body mass index (BMI) (–3,8%), viscerale vetarea (–14,4%) en ontstekingsparemeters (vastende glucose = –18,2%, vast insuline = –50,1%; HOMA = –57,8%). C-reactief eiwit (CRP), als

belangrijkste indicator voor LGI, steeg tot een gemiddelde van 169,6%.

Onze gegevens tonen aan dat het terugkeren naar een Paleolithische levensstijl positieve effecten kan hebben op risicofactoren die vaak verband houden met metabole stoornissen, zoals obesitas en diabetes mellitus type 2. De individuele factoren die verantwoordelijk zijn voor de waargenomen voordelen van onze vier dagen interventie op basis van dieet en levensstijl zijn moeilijk, zo niet onmogelijk, toe te wijzen vanwege de meerdere radicale veranderingen in vergelijking met een geïndustrialiseerde omgeving. Naast caloriebeperking en intermitterend vasten, kan de onvermijdelijk spontane fysieke activiteit voor voedsel en water inname een van de belangrijkste voordelen van onze interventie zijn. Een ander aspect is de volledige afsluiting van gemeenschappelijke stressbronnen in verband met onze moderne levensstijl, zoals tijdsdruk, verkeersruis en visuele complexiteit in ruil voor oude gevaarsignalen (dorst, honger, te hoge of te koude temperaturen) omringd door bebost landschap. Dorst, honger en andere gevarensignalen worden beschouwd als natuurlijke stressoren, die ons voor het grootste deel van onze evolutionaire geschiedenis hebben begeleid. Het is mogelijk dat de synergistische effecten die bij deze interventie in overeenstemming zijn, uiteindelijk de voornaamste veroorzakers zijn voor de veelbelovende resultaten.

Deze bevindingen kunnen leiden tot verder onderzoek om de vraag te beantwoorden of de reeds aanwezige metabolische condities en/of auto-immuun- en neuro-inflammatoire aandoeningen gunstig beïnvloed kunnen worden door een Paleolithische levensstijl.

Invloed van een 10-dagen nabootsing van onze oude levensstijl op antropometrie, metabole parameters van metabolisme en ontsteking: een 'studie naar de oorsprong'

In **Hoofdstuk 1.4** hebben we nog een onderzoek uitgevoerd om te onderzoeken of een 10-daagse imitatie van een jager-verzamelaar levensstijl een positieve invloed heeft op antropometrie en klinisch-chemische uitkomsten. De afwezigheid van oude immuun problemen in de huidige westerse samenlevingen inspireerde ons tot de hypothese dat acute stress door oude gevaarsignalen een herverdeling van het immuunsysteem veroorzaakt, richting evolutionaire voorkeurslocaties. Hierdoor worden de toestand van chronische systemische LGI gunstig beïnvloedt, de activiteiten van stressassen genormaliseerd, ritmische functie herstelt, en insuline-ongevoeligheid verbetert. Lichte stressfactoren kunnen reacties, op basis van overlevingsmechanismen die zijn ontstaan uit miljoenen jaren van evolutionaire druk, activeren. In deze studie onderzochten we of dergelijke ´oude stressoren´, die via een 10-daagse reis door de Pyreneeën werden aangeboden, de antropometrie en verschillende klinische chemische parameters van LGI, stress en metabolische controle verbeterden bij 55 gezonde ogende volwassenen. Het doel was om het principe aan te tonen dat mensen hun immuun- en metabolische systemen kunnen beïnvloeden door blootstelling aan oude milde acute stressfactoren. De interventie in ons onderzoek heeft tot op zekere hoogte de ´bestaansvoorwaarden´ van voorouderlijke en huidige jacht- en visserijpopulaties gemeen.

Vijfenvijftig schijnbaar gezonde deelnemers, verdeeld in vijf groepen, namen deel aan een 10-daagse reis door de Pyreneeën. Ze liepen gemiddeld 14 km per dag, met een acht kilo rugzak. Rauw eten werd verstrekt en zelf bereid, water werd verkregen uit waterputten. Ze sliepen buiten in slaapzakken en werden blootgesteld aan temperaturen variërend van 12 tot 42 °C. Antropometrische gegevens en bloedmonsters werden verzameld aan het begin en eind van de studie.

Wij vonden belangrijke veranderingen in de meeste uitkomsten die antropometrie en metabolische functie bevorderen. Het lichaamsgewicht daalde met een mediaan van -3,8 kg (-12,5 tot -0,7), BMI met -1,2 kg/m^2 (-4,4 tot -0,2), heupomtrek met -3 cm (-17 tot +5), taille omtrek met -5 cm (-18 tot +9) en taille/heup ratio met -0,02 (-0,14 tot +0,10). Ook waren er afnamen in: glucose (-0,6; -1,7 tot +0,5 mmol/l), HbA1c (-0,1; -0,4 tot +0,2%), insuline (-4,7; -31,4 tot -0,2 pmol/L), HOMA-IR (-1,2; -7,0 tot -0,4 mmol*mU/L^2), triglyceriden (-0,14; -6,12 tot + 2,18 mmol/L); totaal cholesterol (TC) 2,1 tot +0,4 mmol/L), LDL-cholesterol (LDL-C) (-0,6; -3,1 tot + 0,6 mmol/L), triglyceriden/HDL-cholesterol (HDL-C) -0,55; -8,98 tot 1,34 mol/mol) en FT3 (-0,8; -3,4 tot +3,1 pmol/L). Anderzijds vonden we dat ASAT- en ALAT-activiteiten met respectievelijk 11 IU/L (-8 tot 54) en 6 IU/L (-13 tot 52) waren toegenomen, terwijl CRP met 0,56 mg was toegenomen/L (15,72 tot +41,07). De ASAT/ALAT-verhouding steeg met 0,08 tot 1,31 (0,48–2,06).

Onze interventies kunnen gebaseerd zijn op 'milde acute stress' bij mensen die in hun dagelijkse leven

blootgesteld worden aan chronische stress, in overeenstemming met onze moderne levensstijl. Acute stress bevordert het vrijkomen van stresshormonen, met inbegrip van adrenaline, noradrenaline en cortisol, die elk een diepgaande metabolische en immunologische aanpassing veroorzaken. Acute stressfactoren verhogen autonome activiteit, versnellen immuun cel proliferatie en differentiatie, en stimuleren ook de anti-inflammatoire component van het immuunsysteem (d.w.z. productie van IL10, lactoferrine, lysozym). Niettemin produceert milde stress in eerste instantie een pro-inflammatoire reactie, die vervolgens kan leiden tot herstel van de regerende toestand van chronische LGI en de terugkeer naar homeostase.

Een korte periode van terugkeer naar de 'bestaansomstandigheden' die vergelijkbaar zijn met die waarop ons genoom is gebaseerd, kan de antropometrie en het metabolisme verbeteren door het immuunsysteem positief uit te dagen bij gezond ogende proefpersonen en mogelijk ook bij patiënten met fibromyalgie. Deze 'terugkeer' kan wel tot een meer actieve ontstekingsreactie leiden, als tegenhanger voor het chronisch systeem LGI dat typisch is voor onze huidige levensstijl van welvaart. We kunnen meer en meer waarderen dat we het niet allemaal tegelijk kunnen hebben, terwijl de evolutionaire lessen van Darwin en interventiestudies ons leren dat preventie meer belonend en betaalbaar kan zijn dan de huidige cultuur van medische behandeling.

Verzadigde vetzuren (SFA)

Er is veel controverse over de invloed van verzadigde vetzuren (SFA) op hart- en vaatziekten (CVD). De huidige opvatting is dat inname van SFA nadelig is en met name palmitische (16:0), laurische (12:0) en myristische (14:0) zuren moeten beperkt worden ingenomen. Palmitinezuur is overvloedig in b.v. dierlijk vet (inclusief moedermelk) en palmolie, terwijl zowel laurische- als myristische zuren vooral overvloedig zijn in kokosnootolie, maar ook in moedermelk.

De relatie van verzadigde vetzuren met lage graad ontsteking (LGI) en hart- en vaatziekten (CVD)

De mantra dat dieet (verzadigd) vet moet worden geminimaliseerd om het CVD-risico te verminderen heeft al decennia voedingsrichtlijnen gedomineerd. In het licht van huidige controverses met betrekking tot hun adequate inname en effecten, beoogt **Hoofdstuk 2.1** onderzoek te doen naar deze heterogene groep vetzuren en de onderliggende mechanismen die verband houden met (chronische) systemische LGI, insulineresistentie, metabolisch syndroom en met name CVD.

De belangstelling voor de relatie tussen voedingsvet en CVD ontstond uit dierstudies, die aangaven dat cholesterol uit voeding arteriële letsels veroorzaakte, dit wordt grotendeels gemedieerd door een verhoging van het bloedcholesterolgehalte. Sindsdien is de relatie tussen dieet- en CVD-risico's intensief bestudeerd, met behulp van verschillende benaderingen, waaronder gecontroleerde voedingsstudies, gerandomiseerde gecontroleerde studies (RCT's) en grote cohortstudies. De meeste studies naar SFA richten zich uitsluitend op de verandering in lipoproteïne metabolisme die SFA veroorzaakt en op het beïnvloeden van de concentraties lipoproteïnen die cholesterol dragen. Echter deze studies laten de vraag wat het gezondste algehele mengsel van de verschillende klassen voedingsvet vormt, onbeantwoord. Het onbetwistbaar hoge SFA-gehalte van menselijke melk getuigd van hun gunstige effecten, in ieder geval bij borstvoeding, onder meer vanwege hun antimicrobiële eigenschappen en als snel beschikbare energiebronnen.

Uit de sterke relatie tussen ontsteking en metabolisme, met inbegrip van het metabolisme van glucose, vet en cholesterol, blijkt dat de dyslipidemie in de westerse samenlevingen beïnvloed wordt door veel ongunstige levensstijl factoren. Dit geldt met name voor verhoogde triglyceriden, ´kleine dicht´ lipoproteïne met een lage dichtheid en ´dysfunctioneel´ lipoproteïne met hoge dichtheid. Zoals Paracelsus (1493-1541) reeds zei: "De dosis (en omstandigheden) bepaalt of het giftig is". Onze voeding is samengesteld uit complexe biologische systemen, zoals vlees, vis, groenten en fruit, waarin de voedingsstoffen, SFA inbegrepen, gehoor geven aan het evenwicht zoals deze in het levende materiaal voorkomt. Op deze balans heeft de mens zijn gezondheid ontwikkeld. Bovendien is het belangrijk om inzicht te krijgen in de interactie tussen de verschillende levensstijl factoren. Dieet SFA is slechts één van de vele levensstijl factoren die een rol spelen in chronisch systemische LGI en de daarbij behorende metabolische aanpassingen, waaronder de veranderingen in concentraties van circulerende lipiden en lipoproteïne-cholesterol. De omgeving biedt ons veel andere pro-inflammatoire stimuli dan SFA, maar ook met veel compenserende anti-inflammatoire stimuli. Het gaat allemaal om 'balans'.

Wij benoemen dat het de interactie is tussen veel levensstijl factoren die bepalen of SFA, en in feite elk nutriënt, bijdraagt tot systemische LGI, veranderingen in lipoproteïne metabolisme en uiteindelijk een verhoogd CVD risico. De afwijking tussen proinflammatoire en anti-inflammatoire stimuli in onze westerse samenleving komt niet uit één enkele oorzaak en kan derhalve ook niet worden opgelost door een enkele 'magische kogel'. Resolutie van het conflict tussen onze zelfgemaakte omgeving en ons oude genoom kan beter gebaseerd worden op de terugkeer naar de levensstijl van het Paleolithische tijdperk volgens de cultuur van de 21ste eeuw. Daarom kunnen we de voedingsrichtlijnen aanbevelingen voor het vervangen van SFA heroverwegen, aangezien ons eten, en niet de voedingsstoffen, de fundamentele eenheid van voeding is. Dieet moet daarom worden onderzocht in een bredere context, samen met niet-dieet gerelateerde levensstijl factoren. Dit zou een duidelijke prioriteit moeten zijn, in tegenstelling tot de reductionistische aanpak om de effecten van enkele voedingsstoffen, zoals SFA, in isolatie te bestuderen.

Opmerkingen toegevoegd aan de SFA review in augustus 2017

In **Hoofdstuk 2.1.a**, zijn we er op basis van de recente 'White Paper' van Pett et al achter gekomen dat figuur 1 in hoofdstuk 2.1 onterecht is toegeschreven aan The Seven Countries Study, opgericht door Ancel Keys. Dit figuur is afgeleid van uit de studie van Yerushalmy en Hilleboe, gepubliceerd een jaar voor aanvang van The Seven Countries Study. Deze studie en zijn bevindingen zijn gebruikt om The Seven Countries Study in de literatuur en sociale media te illustreren, maar ze doen dat eigenlijk niet. Op basis van deze gegevens is Keys vals beschuldigd van *'cherry picking'* om zijn relatie tussen SFA-inname en CVD te maken. Onze figuren 1A en B werden niet in dezelfde studie geproduceerd, en geen van hen was van de Seven Countries Study. Gezien de inherent onbetrouwbare aard van voedselgegevens en mortaliteit voorafgaand aan The Seven Countries Study, blijft echter de vraag welke criteria Keys gebruikt heeft voor de selectie van die zes landen die in het figuur uit 1953 werden vertegenwoordigd.

Ondanks onze bovengenoemde nalatigheid veranderen wij onze mening echter niet over de invloed van dieet SFA op CVD. De 'White Paper' heeft namelijk niet als doel in voedingsadvies te voorzien, maar is uitsluitend bedoeld om "een historisch nauwkeurig verslag van goed gedocumenteerd werk voor te leggen en foutieve voorstellingen van dat werk te herstellen". Waarbij het laatste verwijst naar het werk van Keys als de oprichter van The Seven Countries Study.

Verzadigde vetzuur (SFA) -status en SFA-inname vertonen verschillende relaties met totaal serum cholesterol en lipoproteïne-cholesterol. Een mechanistische verklaring gericht op levensstijl geïnduceerde lage graad van ontsteking (LGI)

De totale cholesterol (TC)/HDL-cholesterol (HDL-C) verhouding is een veel gebruikte CVD-risicofactor. In **Hoofdstuk 2.2** onderzochten we de relaties tussen vetzuurstatus en serum TC, LDL-C, HDL-C en de TC/HDL-C-verhouding in vijf Tanzaniaanse etnische groepen en één Nederlandse groep, en onderzochten we of deze correlaties verschillen van de gerapporteerde effecten van SFA-inname in westerse samenlevingen. De onderzochte etnische groepen verschillen sterk in de inname van zowel SFA als LA. Vetzuurstatus werd bepaald door meting van vetzuren in serumcholesterolesters en erytrocyten (RBC). Gegevens die de invloed van vetzuurinname op het totale cholesterol en lipoproteïnecholesterol weerspiegelen werden verkregen uit eerder uitgevoerde gedocumenteerde interventiestudies.

De inwoners van het eiland Chole hebben een hoge inname van lokale zeevis en kokosnoot en consumeren veel vrijgroeiend fruit en groenten. Ze gebruiken geen plantaardige oliën om te koken en hebben weinig inname van koolhydraten (CHO) uit granen of mais. Zowel de Maasai, Ruvu en Wasso diëten bestaan uit melk en vlees uit hun eigen voorraad, en is onlangs aangevuld met *ugali* (maïspap). Het verbruik van het volledige dier karkas is een regelmatige praktijk onder de Maasai. Vissen worden meestal niet gegeten, terwijl groenten en fruit als voedsel voor koeien worden beschouwd. De mensen uit Sengerema hebben een regelmatige visinname (gemiddeld 4-5 keer per week). *Ugali, muhogo* (cassave wortel) en plantain (gebakken banaan) zijn veel gegeten voedsel. De Hadzabe zijn traditionele jager-verzamelaars waarvan het dieet bestaat uit bessen, wortels, honing, vlees en af en toe vis. Ze jagen op kleine dieren in het natte seizoen en groter wild in het droge seizoen. In de afgelopen jaren zijn maïs- en maïsolie onvermijdelijk een groot deel van hun hedendaagse dieet geworden. De vet inname

in Nederland bedraagt ongeveer 34 en%, en is na CHO de belangrijkste energiebron. Gemiddelde SFA, mono-onverzadigde vetzuur (MUFA) en meervoudig onverzadigde vetzuur (PUFA) inname zijn respectievelijk 12,5, 12,7 en 6,3 en%. Zuivelproducten, vlees en vleesproducten, vet en gebak zijn de belangrijkste bronnen van dieetvet en SFA in Nederland. Graan, graanproducten en alcoholvrije dranken zijn de belangrijkste bronnen van dieet CHO. Meer dan 75% van de huidige Nederlandse volksgezondheid voldoet niet aan de aanbeveling van consumptie van 200 g fruit en 200 g groenten per dag, noch de consumptie van één keer vis per week (in het bijzonder vette vis) bij volwassenen. In feite bedraagt het gemiddelde visverbruik nauwelijks drie keer per maand, de inname van zout wordt te veel beschouwd en zo ook de inname van CHO met een hoog glycemische index.

We vonden dat de 14:0-, 16:0- en SFA-status, maar niet hun inname, positief correleren met de TC/HDL-C-verhouding. LA- en PUFA-status en PUFA-inname vertoonden negatieve relaties met de TC/HDL-C-verhouding. Op basis van de TC/HDLC- verhouding, de vaak gebruikte CVD-risicofactor, vonden we dat: 1) op basis van de FA-status 14: 0, 16: 0 en SFA nadelige effecten hebben, terwijl de 14:0-, 16:0- en SFA-innames neutraal zijn; 2) dat zowel de 18:0-status als de 18:0-inname neutraal zijn; 3) dat de MUFA-status neutraal is, terwijl de MUFA-inname gunstig is; 4) dat de LA- en PUFA-status gunstig zijn net zoals de PUFA-inname; en ten slotte, 5) dat de EPA-status nadelig is.

Onze gegevens suggereren dat, gebaseerd op de TC/HDLC-verhouding, een hoge SFA-status in plaats van een hoge SFA-inname, geassocieerd wordt met verhoogd CVD-risico, terwijl zowel de hoge LA-status als de PUFA-status geassocieerd zijn met een verminderde CVD-risico. Hieruit blijkt dat de TC/ HDL-C-verhouding een twijfelachtige risicomarker is, aangezien meta-analyses van RCT's tonen dat de gedeeltelijke dieetvervanging van SFA voor LA, de dominerende dieet PUFA, het CVD-risico niet verandert. Deze vervanging heeft een net niet significant effect op hogere sterfte als gevolg van CVD (hazard ratio 1,33; 95% confidence limit 0,99–1,79). We concluderen dat veel levensstijlfactoren, niet alleen SFA-inname, de SFA-status bepalen en suggereren dat de interactie met andere levensstijlfactoren bepaalt of de SFA-status een relevant bijdragend effect heeft op LGI, lipoproteïneveranderingen en CVD-risico. Uit deze uitkomsten leren we om de gezondheidseffecten van het hele dieet samen met veel niet-dieetstijlfactoren te overwegen, in plaats van een reductionistische aanpak toe te passen om de effecten van enkele voedingsstoffen, SFA en PUFA te bestuderen.

Commentaar op het rapport ´Dietary Fats and Cardiovascular Disease: A Presidential Advisory from the American Heart Association (AHA)´

Onlangs publiceerde de American Heart Association (AHA) een meta-analyse die de nadruk legde op hun eerdere aanbeveling om de inname van SFA te beperken. SFA moet vervangen worden door onverzadigd vet, in het bijzonder PUFA, om de incidentie van hartaandoeningen te verlagen. Een dergelijke vervanging zou het risico op hart- en vaatziekten moeten verminderen met ongeveer 30%; een risicovermindering vergelijkbaar met behandeling met statines. De AHA adviseert ook tegen het gebruik van kokosnootolie omdat het LDL-C verhoogt en geen bekende compenserende gunstige effecten heeft. We betogen dat de LDL-C-concentratie nog steeds een zacht eindpunt is, geen ziekte, terwijl er geen studies zijn die ongunstige effecten van kokosnootolie op harde eindpunten aantonen. De AHA motiveert wel de exclusiecriteria van studies voor hun meta-analyse, maar past geen strikte criteria toe bij de inclusie van de vier proeven die de ruggengraat vormen van hun uiteindelijke meta-analyse. Een van deze was geen gerandomiseerd en gecontroleerd experiment, terwijl een andere aan *´performance bias'* leed. De grootste negatieve proef werd onder meer uitgesloten omdat het niet minstens twee jaar liep. De AHA meta-analyse lijkt hiermee bij te dragen aan '*cherry picking*'. Er zijn op dit moment ten minste negen expert review artikelen die geen duidelijke band hebben gevonden tussen SFA, cardiovasculaire sterfte en totale sterfte. We betogen dat individuen met het metabolische syndroom voorzichtig moeten zijn met SFA inname en CHO, aangezien ze SFA *de novo* synthetiseren uit koolhydraten en extra voedingssupplementen. Het hoge risico van personen met het metabolische syndroom is geen reden om de SFA-inname van de werkelijk gezonde populatie te beperken. Sommige SFA's zijn zeker pro-inflammatoir, maar een evenwichtig dieet bevat ook anti-inflammatoire componenten.

Astaxanthine, de roze carotenoïde

Hoofdstuk 3 richt zich op antioxidanten, waarvan specifiek op astaxanthine. Astaxanthine is een unieke

carotenoïde van overwegend mariene oorsprong. De natuurlijke vorm functioneert als een antioxidant zonder pro-oxidante eigenschappen of bijwerkingen na orale inname. Astaxanthine behoort tot de xanthophylfamilie, die de rozerode kleur geeft aan bepaalde microalgen (d.w.z. *Haematococcus pluvialis*) en verzamelt zich in verschillende dieren die hoger zijn in de voedselketen, zoals flamingo's, zalm, garnalen en crayfish. Het is waarschijnlijk dat astaxanthine deel uitmaakt van het land-water-ecosysteem waaruit Homo sapiens afkomstig is. Het molecuul strekt zich uit over de fosfolipide dubbele laag van celmembranen als gevolg van zijn twee polaire hoofdgroepen die tussen een vertakte koolstofatoomketen bestaan die negen geconjugeerde dubbele bindingen bevatten. Het is aangetoond dat astaxanthine de immuunrespons verbetert en oxidatieve schade-gerelateerde symptomen vermindert, tevens is het effectief gebleken voor verschillende ziekten en aandoeningen, zoals de ziekte van Alzheimer, obesitas, astma, vergrote prostaat, artrose en reumatoïde artritis.

Kinetiek van plasma- en erytrocyt-astaxanthine bij gezonde proefpersonen na zowel een eenmalige als een onderhouden orale dosis

Hoofdstuk 3.1 presenteert de kinetiek van astaxanthine bij gezonde proefpersonen. Na de inname is eerder aangetoond dat het ongeveer 7 uur duurt voordat astaxanthine een piek bereikt in plasma en vervolgens afneemt met een gemiddelde halveringstijd van ongeveer 21 uur. We onderzochten astaxanthine kinetiek in plasma en erytrocyten (RBC) van vier gezonde volwassenen na een enkele orale dosering van 40 mg. Plasma- en RBC-astaxanthine werden gedurende 72 uur gemeten. Vervolgens werd een dosis van 8 mg/dag gedurende 17 dagen gegeven. Plasma- en RBC-astaxanthine werden elke ochtend gemeten.

Plasma-astaxanthine bereikte na 8 uur een piek (van 79 tot 315 nmol/L) en nam daarna weer af (halveringstijd, 18 uur). Binnen 72 uur was plasma-astaxanthine teruggekomen naar de basislijn. RBC-astaxanthine bereikt een piek (van 63 tot 137 nmol/L-verpakte cellen) na 12 uur en verdwijnt vervolgens (halveringstijd, 28 uur). Tijdens de dagelijkse dosering van 17 dagen verhoogde plasma-astaxanthine tot dag 10 (187 nmol/L) en daalde vervolgens tot een stabiele concentratie die overeenkomt met die bereikt na twee dagen. RBC-astaxanthine bleek zeer variabel te zijn (groepsmediumconcentratie, 86 nmol/L-verpakte cellen).

We vonden hoge intra- en interindividuele variaties, vooral in RBC, mogelijk door het niet-gestandaardiseerd tijdverschil tussen astaxanthine-inname en monsterneming, fluctuerende achtergrondiname van voeding, variabele biologische beschikbaarheid, groot distributievolume, en andere mogelijkheden. Orale astaxanthine wordt snel geabsorbeerd en opgenomen in RBC. De korte plasma- en RBC-astaxanthinehalfwaarden van respectievelijk 18 en 28 uur suggereren dat de dagelijkse astaxanthine noodzakelijk is om een stabiele toestand van meer dan de basiswaarde te handhaven, althans in de aanvankelijke fase van de toevoeging wanneer het totaal lichamelijk evenwicht, indien aanwezig, nog niet is bereikt. Deze vroege fase kan gedeeltelijk beïnvloed worden door de neiging van astaxanthine om in alle lichamelijke celmembranen op te worden genomen.

Aanvulling van astaxanthine bij patiënten met sikkelcelziekte verhoogt plasma- en erythrocyt-astaxanthine en kan de hemolytische component van deze ziekte verbeteren

Sikkelcelziekte (SCD) is een heterogene stoornis die mechanistisch wordt gekenmerkt door hemolytische en vaso-occlusieve componenten. Dit laatste leidt tot cumulatieve ischemische orgaanschade die soms zorgt voor pijnlijke vaso-occlusieve crises. Het geheel van complicaties draagt bij aan een verminderde kwaliteit van leven en vroege dood. De hemolytische component kan geïnitieerd worden door de opwekking van ROS door sikkelhemoglobine (HbS), dicht bij het lipideperoxidatiegevoelige RBC-membraan, waardoor hemolyse ontstaat als dit niet op juiste wijze wordt tegengegaan. De vaso-occlusieve component kan grotendeels worden aangedreven door de hiervoor genoemde hemolytische component. Het behandelen van de hemolytische component, en met name oxidatieve stress, door verbetering van het verwoestende vaso-occlusieve component, lijkt een logische interventiestrategie. Verschillende proeven met natuurlijk voorkomende antioxidanten met veelbelovende resultaten zijn gerapporteerd, waaronder die met vitamine E, curcuminoïden, gerijpt knoflook extract, N-acetylcysteïne en zink. Door de unieke antioxidant eigenschappen kan astaxanthine supplementatie de hemolytische component van SCD vergemakkelijken.

Hoofdstuk 3.2 presenteert een open pilot interventie waarbij we 10 laboratorium-bevestigde SCD-patiënten hebben geïncludeerd (zeven volwassenen, drie kinderen, waarvan 3 mannen en 7 vrouwen,

gemiddelde leeftijd 31 jaar, bereik 6-52 jaar) uit het Sint Maarten Medisch Centrum. Wij onderzoeken het effect van een dagelijkse astaxanthine dosering van 8-12 mg orale inname gedurende drie maanden, op plasma- en RBC-astaxanthine-niveaus (primair doel) en verschillende hematologische en klinische chemische parameters (secundair doel), inclusief reticulocytentelling, gemiddelde corpusculaire volume (MCV), RBC distributie breedte (RDW), lactaat dehydrogenase (LDH) en asymmetrische dimethylarginine (ADMA).

Astaxanthine in basislijnplasma (33 nmol/L) en RBC (11 nmol/L-verpakte RBC) nam toe tot 225, 174, 167 nmol/L (plasma) en 149, 100, 71 nmol/L-verpakt RBC naar respectievelijk 1, 2 en 3 maanden. Reticulocyten daalden van baseline en 2 maanden (9,5 en 8,8%) tot 3 maanden (5,6%), MCV van 2 tot 3 maanden (88 tot 86 fL), MCH van baseline tot 3 maanden (30 tot 28 pg) en RDW vanaf baseline na 2 maanden (19,2 en 19,0%) en tot 3 maanden (16,7%). Plasma arginine daalde van 2 tot 3 maanden (46,6 tot 39,4 µmol/L). Astaxanthine supplementatie veranderde geen homoarginine, ADMA, symmetrische dimethylarginine (SDMA) en de ADMA/arginine verhouding. Reticulocyten bij basislijn correleerde met relatieve veranderingen in reticulocyten vanaf basislijn tot 3 maanden. Relatieve veranderingen in reticulocyten correleerden met relatieve veranderingen in RBC, RDW, LDH, ALAT, maar niet hematocrit, binnen dezelfde periode.

We hebben geconcludeerd dat astaxanthine in de behandeling van SCD RBC past en de hemolytische component gunstig kan beïnvloeden. We hebben na 3 maanden een lichte vermindering van de reticulocytentelling gevonden, wat waarschijnlijk een lagere hemolyse aangeeft. Bovendien gaven veel van de patiënten in deze niet-placebogecontroleerde, niet-gerandomiseerde trial aan dat ze zich beter voelden. Een grotere gerandomiseerde gecontroleerde trial wordt aangeraden, waarbij een vergelijkbare of hogere dosis wordt gebruikt, bij voorkeur gedurende meer dan 3 maanden. Het kan zelfs beter zijn om astaxanthine in een aanvullende mix te plaatsen met andere antioxidanten (bijvoorbeeld vitamine E, bètacaroteen, vitamine C en foliumzuur), mineralen (indien nodig selenium, en met name zink); aminozuren (arginine), visolie en vitamine D. Antioxidanten werken niet alleen maar zijn nogal onderdeel van een nog steeds slecht begrepen antioxidantnetwerk van vrije radicalen, *quenchers* en antioxidante enzymen en daarom lijkt het onwaarschijnlijk een enkele ´magische kogel´ te vinden om ziekte die verband houdt met oxidatieve stress te voorkomen of te behandelen.

Hogere prevalentie van 'laag-T3 syndroom' bij patiënten met chronisch vermoeidheidssyndroom: een case-control studie

Chronisch-vermoeidheidssyndroom (CFS) is een heterogene ziekte met onbekende oorzaak(en). Veel pathofysiologische cascades zijn verondersteld, maar onderliggende organische omstandigheden worden zelden gevonden. Een verstoorde hypothalamus-hypofyse-adrenale (HPA) as, gepresenteerd als mild hypocortisolisme, verhoogde negatieve feedback en afgestompte responsen op uitdaging zijn gevonden in CFS. CFS-symptomen lijken op een hypothyreoïdie toestand, mogelijk afhankelijk van chronische (lage graad) (metabolische) ontsteking. We hebben misschien te maken met een menselijk equivalent van 'hibernatie' met oorzaken geworteld in de typische westerse levensstijl die LGI veroorzaakt.

In **Hoofdstuk 4** presenteren we een CFS case-control studie. We hebben 98 CFS-patiënten (21-69 jaar, 21 mannen) en 99 leeftijds- en geslacht gekoppelde controles (19-65 jaar, 23 mannen) onderzocht. Wij hebben daarbij parameters gemeten van schildklierfunctie, (metabolische) ontsteking, integriteit van de darmwand en voedingsstoffen die schildklierfunctie en/of ontsteking beïnvloeden.

Als meest opmerkelijke resultaat, vertoonden CFS-patiënten soortgelijke TSH, maar lagere vrije T3 (FT3) (verschil van medianen 0,1%), totaal T4 (TT4) (11,9%), totaal T3 (TT3) (12,5%) en %TT3 (4,7%), hogere % omgekeerde T3 (rT3) (13,3%) en lager 24-urine jodium (27,6%). FT3 onder het referentiebereik, in overeenstemming met het 'low T3 syndroom', werd gevonden in 16/98 CFS patiënten versus 7/99 controles (OR 2,56; 95% CI = 1,00–6,54). De meeste waarnemingen bleven in twee gevoeligheidsanalyses met strengere afsnijwaarden voor body mass index, hoog gevoelig CRP (hsCRP) en witte bloedcellen. We hebben mogelijk bewijs gevonden van (chronische) lage graad metabolische ontsteking (ferritine en HDL-cholesterol). FT3, TT3, TT4 en rT3 correleren positief met hsCRP bij CFS-patiënten en alle deelnemers. Alleen TT3 en TT4 correleren positief met hsCRP in controles.

Lage circulerende T3 en de schijnbare verschuiving van T3 tot rT3 kunnen meer ernstig aangedaan weefsel T3 niveaus weerspiegelen. De huidige bevindingen kunnen in lijn zijn met recente metabolische

studies die naar een hypometabolische toestand wijzen. Ze lijken op een zachte vorm van *'non-thyroidal syndrome'* en het 'low T3 syndroom' ervaren door een subgroep hypothyroid patiënten die T4 monotherapie krijgen. Onze studie heeft bevestiging en verlenging door toekomstige studies nodig. Indien bevestigd, kunnen proeven met b.v. T3 en jodide supplementen worden gedaan. CFS is waarschijnlijk een heterogene ziekte met een gemeenschappelijke definitieve pathofysiologische route. De huidige bevindingen zijn mogelijk in lijn met een gemeenschappelijke definitieve weg, maar brengen ons nog niet dichter bij de oorzaak(en).

Epiloog. Toepasbaarheid van een evolutionaire aanpak bij (chronische) ziekten en preventie

'Evolutionaire Geneeskunde' krijgt acceptatie. Het invloedrijke tijdschrift 'The Lancet' heeft onlangs drie artikelen gepubliceerd en een redactioneel stuk over dit onderwerp gepubliceerd [1–4]. Wat logica reeds voorspelde laat de wetenschap nu ook zien: Lifestyle, niet Genetica, is de belangrijkste belangrijke oorzaak van de 'typisch westerse' welvaart ziekten [5].

In dit proefschrift hopen we te hebben bijgedragen aan het bovenstaande concept, en meer specifiek op het bewustzijn dat *'healthy aging'* een levensstijl is. De componenten van een ongezonde levensstijl zijn onevenwichtig dieet, onvoldoende lichamelijke activiteit, onvoldoende slaap, chronische stress, abnormale microbiële flora (microbioma) en schadelijke omgeving (bijvoorbeeld rook, fijn stof). Vanwege hun interactie is het waarschijnlijk niet zo productief om deze risicofactoren alleen in isolatie te bestuderen, ongeacht of het over gerandomiseerde gecontroleerde trials (RCT's) gaat.

Er is en is nog steeds weinig aandacht voor de beperkingen van RCT's en nog minder voor de beperkingen van de meta-analyses daarvan. Rawlins [6] merkt op dat: "Hierarchieën van bewijzen moeten worden vervangen door een verscheidenheid aan benaderingen te accepteren – beter nog te omarmen". Niettemin worden RCT's en hun meta-analyses algemeen beschouwd als het 'hoogste niveau van bewijs', en zijn ze volgens sommigen zelfs 'de enige basis voor het maken van richtlijnen'. Deze benadering, die mogelijk het meest geschikt is voor medicatie, maar niet noodzakelijkerwijs voor voedingsstoffen, heeft de huidige status van een 'paradigma' bereikt, gedefinieerd als 'een gedeeld begrip tussen wetenschappers of geleerden die in een discipline werken in verband met de belangrijke problemen, structuren, waarden en aannames die bepalend zijn die discipline' [7].

Van begin af aan waren RCT's en hun meta-analyses niet bedoeld om de enige componenten van 'Bewijs-gebaseerde Geneeskunde/Voeding' te vormen, althans niet in de gedachten van de oorspronkelijke uitvinders [8, 9]. Bovendien komen de huidige meta-analyses vaak met tegenovergestelde conclusies, zelfs na het lezen van precies dezelfde literatuur. Een voorbeeld is de recente op meta-analyse gebaseerde aanbeveling voor verzadigd vet afgegeven door de AHA, die tegen gesteld is aan "ten minste 17 meta-analyses en systematische reviews en vijf niet-systematische reviews die geen duidelijke band hebben gevonden tussen verzadigde vetten en hartziekten/hart- en vaatziekten" [10]. Het selectieve weglaten van studies door 'slechte kwaliteit', eventueel geïnspireerd door vooroordeel, ook wel *'cherry picking'* genoemd, zou de nieuwe trend kunnen zijn geworden en staat thans in het middelpunt van de discussie [11–13].

Dergelijke controverses geven geen groot vertrouwen in de wetenschap en frustreren de publieke opinie. Tegenwoordig hebben zowel geleerde niet-insiders als leken vrijwel onbeperkt toegang tot wetenschappelijke literatuur, terwijl elke 'wetenschappelijke opinie' niet vanzelfsprekend moet worden genomen, zelfs afkomstig uit b.v. een gezaghebbende wetenschapper (ook wel *Eminence Based Medicine* genoemd), Gezondheidsraad of Regering.

Wat tegenwoordig (het grootste deel van) de gemeenschap en (de meeste) regeringen begrijpt is het concept van 'duurzaamheid', gedefinieerd in de ecologie als 'het eigendom van biologische systemen om onbepaald en productief te blijven' [14]. Nu de gevolgen van b.v. de opwarming van de aarde begint te manifesteren, worden er steeds meer prangende vragen gesteld zoals 'wat is er in mijn eten', 'hoe wordt het geproduceerd' en 'waarom herkent mijn grootmoeder dit niet als eetbaar'? De samenleving begrijpt, misschien zelfs beter dan veel wetenschappers, dat we een conflict hebben gecreëerd tussen onze omgeving en ons oude genoom. Deze laatste kan niet, zowel genetisch (langdurig), noch epigenetisch (kortere termijnen) aanpassen aan de milieu-uitdagingen die zich in een steeds toenemend tempo voordoen. De controverse is niet alleen te wijten aan de hedendaagse beschuldiging van *´bloggers´* [15–18], en gaat niet alleen over verzadigd vet (als één van de onderwerpen in

dit proefschrift) maar ook onder andere koolhydraten, linoolzuur, zout en vitamine D.

Gezien het bovenstaande concluderen we dat de enige effectieve manier om te komen tot '*healthy aging*' het terugkeren is naar de levensstijl van het Paleolithische tijdperk volgens de cultuur van de 21ste eeuw. En dat is precies waar 'Evolutionaire Geneeskunde' over gaat.

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Resumen y Epílogo

Estilo de vida y desequilibrios nutricionales asociados con las enfermedades occidentales

El estilo de vida, y no la genética, podría ser la única y más importante causa primaria de las enfermedades ´típicas occidentales´ de la abundancia. Un estilo de vida insalubre es multifactorial. Fuertes contribuyentes a este estilo de vida insalubre son una dieta desequilibrada, actividad física insuficiente; pobre calidad del sueño, estrés crónico, flora microbiana anormal (microbioma) y ambiente nocivo (por ejemplo, fumar, polvo fino). Estos factores interactúan entre ellos, pero están siendo investigados hoy en día mayoritariamente de manera aislada debido a los erróneos conceptos reinantes sobre ´Medicina Basada en Evidencia´ y ´Nutrición Basada en Evidencia´. Este primer capítulo tiene como objetivo abordar el estilo de vida desde un punto de vista holístico, en vez de desde una investigación reduccionista de sus componentes aislados.

Estilo de vida y desequilibrios nutricionales asociados con las enfermedades occidentales: causas y consecuencias de inflamación de bajo grado crónica y sistémica en un contexto evolutivo

Con la llegada de las revoluciones Agrícola e Industrial, hemos introducido un gran número de falsos desencadenantes de inflamación en nuestro estilo de vida, llevándonos hacia un estado de inflamación de bajo grado (LGI) crónica y sistémica que finalmente conduce a las enfermedades típicas occidentales mediante una interacción evolutivamente conservada entre nuestro sistema inmune y el metabolismo. La alteración de nuestro equilibro inflamatorio/anti-inflamatorio se ilustra en este capítulo por medio de ácidos grasos y antioxidantes en la alimentación. El descenso actual en años sin enfermedad crónica es más adquirido (*´nurture´*) que innato (*´nature´*), ya que menos del 5% de las típicas enfermedades occidentales son en origen atribuibles a factores genéticos.

En el **Capítulo 1.1.** nos centramos en los cambios de estilo de vida, especialmente hábitos dietéticos, que están en la base de la LGI crónica y sistémica, resistencia a la insulina y enfermedades occidentales centradas alrededor del síndrome metabólico. El síndrome metabólico es la combinación de grasa abdominal excesiva, homeostasis de glucosa alterada, hipertensión y dislipidemia aterogénica (el ´cuarteto mortal´). Constituye un riesgo para diabetes mellitus tipo 2, enfermedad cardiovascular (ECV), algunos cánceres, enfermedades neurodegenerativas (por ejemplo, enfermedad de Alzheimer), embarazo, problemas de fertilidad y otras enfermedades. La inflamación sistémica causa resistencia a la insulina y una hiperinsulinemia compensadora que se esfuerza en mantener en equilibrio la homeostasis de glucosa. El objetivo de la sensibilidad a la insulina reducida es, entre otros, la reasignación de los nutrientes ricos en energía debido a un sistema inmune activado, y la reparación del daño infringido.

La sensibilidad del *homo sapiens* para desarrollar resistencia a la insulina se remonta a nuestro rápido crecimiento de cerebro hace 2,5 millones de años. Una reacción inflamatoria compromete las elevadas necesidades de glucosa de nuestro cerebro, causando varias adaptaciones, incluyendo resistencia a la insulina, reasignación funcional de los nutrientes ricos en energía, y cambios en la composición de las lipoproteínas séricas. Estos últimos tienen como objetivo la redistribución de los lípidos, la modulación de la reacción inmune y la inhibición activa del transporte reverso de colesterol para la reparación del daño. La resolución del conflicto entre el medio ambiente y nuestro genoma ancestral podría ser la única manera efectiva de llegar a un ´envejecimiento saludable´, y para alcanzar este objetivo quizás deberíamos volver al estilo de vida de la era Paleolítica según la cultura del siglo XXI.

Los pacientes sometidos a injerto electivo de bypass coronario (CABG) presentan pobres ingestas pre-operativas de frutas, verduras, fibra alimenticia, pescado y vitamina D

La ECV puede ser el resultado de LGI crónica y sistémica. En ambas, la dieta es un factor de riesgo modificable y su optimización podría reducir la mortalidad postoperatoria, la fibrilación atrial y el deterioro cognitivo. En el **Capítulo 1.2.** investigamos las ingestas habituales de pacientes sometidos a injerto electivo de bypass coronario (CABG) haciendo especial énfasis en los grupos de comida y nutrientes con roles putativos en el equilibrio inflamatorio/anti-inflamatorio. Las dietas pobres en ácidos grasos ω3, antioxidantes naturales, fibra alimenticia, y frutas y verduras, están entre los factores pro-inflamatorios en nuestra dieta, y lo mismo se aplica a valores bajos de vitamina D.

Desde noviembre de 2012 a abril de 2013

contactamos con 93 pacientes consecutivos (80% hombres) sometidos a CABG, de los cuales fueron finalmente incluidos 55 (46 hombres, 9 mujeres). Para la estimación de las ingestas nutricionales usamos un cuestionario de frecuencia de comidas (FFQ) de la División de Nutrición Humana de la Universidad de Wageningen, Países Bajos. El FFQ elaborado se evaluó usando la media de tres recordatorios de 24 horas como método de referencia, en el cual se encontraron asociaciones razonables para vitamina D, fibra alimenticia, verduras y frutas. Para el mismo FFQ, se encontraron asociaciones muy fuertes para ingesta de energía. A los pacientes se les pidió que informaran sobre sus hábitos dietéticos en las cuatro semanas anteriores.

El índice de masa corporal (IMC) medio fue de 27 (rango: 18–36) kg/m^2. Las ingestas (mediana; rango) fueron: frutas (181; 0–433 g/día), verduras (115; 0–303 g/día), fibra alimenticia (22; 9–45 g/día), ácido eicosapentaenoico (EPA) + ácido docosahexaenoico (DHA) (0,14; 0,01–1,06 g/día), vitamina D (4,9; 1,9–11,2 µg/día), grasa saturada (SFA) (13,1; 9–23% energía) y ácido linoleico (LA) (6,3; 1,9–11,3% energía). Los porcentajes de pacientes con ingestas por debajo de las recomendaciones fueron: 62% (frutas; recomendación: 200 g/día), 87% (verduras; recomendación: 150–200 g/día), 73% (fibra alimenticia; recomendación: 30–45 g/día), 91% (EPA+DHA; recomendación: 0,45 g/día), 98% (vitamina D; recomendación: 10–20 µg/día) y 13% (LA; recomendación: 5–10 %energía). Los porcentajes por encima de las recomendaciones fueron: 95% (SFA; recomendación: <10% energía) y 7% (LA). Excepto un paciente (3% de la población de estudio por debajo de 70 años, 2% de la población total de estudio), ninguno de ellos informó de una ingesta a partir de la comida de vitamina D (mediana 4,9 µg/día, rango 1,9–11,2) que alcanzase las necesidades diarias, mientras que ninguno de ellos cumplió con las recomendaciones americanas (para sujetos no expuestos a la luz solar: 15 µg/día para 1–69 años, y 20 µg/día para ≥70 años). Excepto tres pacientes (5%; dosis desconocida), ninguno de ellos informó del uso de un suplemento de vitamina D.

Encontramos que las ingestas dietéticas de los pacientes podían compararse con la ingesta nutricional media de sus correspondientes en edad y sexo de la población sana holandesa. Por ejemplo, las recomendaciones nutricionales de la Asociación Americana del Corazón (AHA) son de al menos dos raciones de pescado a la semana (particularmente pescado azul), mientras la recomendación nutricional holandesa es de únicamente una a la semana, traducido a 0,45 g de EPA+DHA. De todos modos, en 1998, el consumo medio de pescado en los Países Bajos ascendía a duras penas a tres veces al mes. Estas dietas pre-operativas desequilibradas podrían poner a los pacientes en riesgo de resultados quirúrgicos desfavorables, ya que promueven un estado pro-inflamatorio. Concluimos que hay una necesidad urgente de estudios de intervención con el objetivo de mejorar rápidamente sus dietas para reducir los riesgos peri-operativos. Dependiendo del peso corporal, estas intervenciones deberían, en nuestra opinión, ir encaminadas hacia dietas iso- o hipo-calóricas, con ingesta moderada de carbohidratos (por ejemplo, 40% energía), proteínas moderadas-altas (por ejemplo, 25% energía), grasas moderadas (35% energía) y suplementación con vitamina D y B_{12}. Esto se traduce en una dieta de carga glicémica baja, rica en fibra y abundante en micronutrientes y fitoquímicos procedentes de verduras, frutas y frutos secos, junto con carne magra y pescado rico en EPA+DHA.

Para reestablecer la salud ´¿tenemos que volver al futuro?´ El impacto de un cambio de estilo de vida Paleolítico durante 4 días sobre el metabolismo humano – estudio piloto

En el camino desde la Edad de Piedra hacia las condiciones de alta tecnología actuales *via* Revolución Agrícola, los humanos han perdido su conducta primitiva de búsqueda de alimento. Hoy en día, el gasto energético ya no es necesario para cazar ni recolectar, y las enfermedades metabólicas están alzándose de manera epidémica allá donde nuestro estilo de vida original Paleolítico está cambiando hacia un estilo de vida sedentario moderno.

En el **Capítulo 1.3**, examinamos si, comparado con el estado inicial de base, los cambios de estilo de vida hacia un patrón más de estilo Paleolítico por un período de cuatro días se correlacionaban con cambios en una variedad de parámetros metabólicos usando un diseño intra-participante. En este estudio piloto, nos basamos en el concepto de que un cambio radical hacia un estilo de vida del cazador-recolector Paleolítico podría servir como estrategia terapéutica contra cualquier enfermedad meta-inflamatoria, incluso a corto plazo. Trece voluntarios adultos sanos fueron trasladados al Parque Nacional DELUX (Alemania y Luxemburgo) durante cuatro días y tres noches, donde se imitaron situaciones de la Edad de Piedra.

Se midieron 38 parámetros bioquímicos y bioeléctricos en los participantes antes y después de esta reubicación. Hubo descensos significativos en peso corporal (–3,9%), grasa corporal (–7,5%), IMC (–3,8%), área de grasa visceral (–14,4%) y parámetros relacionados con meta-inflamación (glucosa en ayunas = –18,2%; insulina en ayunas = –50,1%; HOMA = –57,8%). La proteína C-reactiva (PCR), como indicador principal de LGI, aumentó hasta una media de un 169,6 %.

Nuestros datos muestran que volver a nuestras raíces del Paleolítico podría tener efectos positivos en factores de riesgo comúnmente asociados a desórdenes metabólicos tales como obesidad y diabetes mellitus tipo 2. Los factores individuales responsables de los beneficios observados en nuestra inmersión de cuatro días en los cimientos evolutivos de la dieta y estilo de vida son difíciles, si no imposibles, de localizar, debido a los múltiples cambios radicales comparados con un medio ambiente industrializado. Además de restricción calórica y ayuno intermitente, la actividad física inevitable y espontánea previa a la ingesta de comida y agua podría ser uno de los principales factores beneficiosos en nuestra intervención. Otro aspecto es la completa desconexión de las fuentes habituales de estrés asociadas a nuestro estilo de vida moderno, tales como la presión de tiempo, el ruido del tráfico y la complejidad visual, canjeadas por viejas señales de peligro (sed, hambre, temperaturas muy altas o muy bajas) rodeadas de paisaje arbolado. Considerados estresores naturales, la sed, el hambre y otras señales de peligro nos han acompañado durante la mayor parte de nuestra historia evolutiva. Podría ser posible que, en última instancia, los efectos sinérgicos que concurren en esta intervención sean los conductores principales responsables de tales resultados prometedores.

Estos hallazgos podrían liderar el camino a más investigación para contestar la pregunta de si las condiciones metabólicas previamente existentes y/o las enfermedades autoinmunes y neuro-inflamatorias podrían ser afectadas de manera favorable por un estilo de vida Paleolítico.

Influencia de una simulación de 10 días de nuestro estilo de vida ancestral de duración en valores de antropometría y parámetros de metabolismo e inflamación: el ´Estudio del Origen´

En el **Capítulo 1.4** llevamos a cabo otro estudio piloto para investigar si una simulación de 10 días de un estilo de vida de cazador-recolector afecta favorablemente a valores de antropometría e índices clínicos químicos. La ausencia de desafíos inmunes antiguos en las sociedades occidentales actuales nos inspiró para hipotetizar que el estrés agudo procedente de las señales de peligro ancestrales causa una redistribución del sistema inmune hacia sus localizaciones preferidas evolutivamente y, por tanto, afecta favorablemente al estado de LGI crónica sistémica, normaliza la actividad de los ejes de estrés, recupera la función rítmica y recupera los caminos con insensibilidad a la insulina. Los factores de estrés moderado podrían activar las respuestas de resolución basadas en mecanismos de supervivencia que se originan a partir de millones de años de presión evolutiva. En este estudio, investigamos si tales ´estresores ancestrales´, proporcionados a través de un viaje de 10 días por los Pirineos, mejoraron valores de antropometría y varios parámetros químicos clínicos de LGI, estrés y control metabólico en 55 adultos aparentemente sanos. El objetivo era el de proporcionar un estudio preliminar de eficacia por medio del concepto de que los humanos pueden influir en su sistema inmune y metabólico mediante la exposición a factores ancestrales de estrés agudo moderado. La intervención en nuestro estudio piloto simuló, hasta cierto punto, las ´condiciones de existencia´ de las poblaciones de cazadores/pescadores-recolectores ancestrales y actuales.

Cincuenta y cinco sujetos aparentemente sanos, en cinco grupos, se comprometieron a un viaje de 10 días a través de los Pirineos. Caminaron una media de 14 km/día llevando una mochila de ocho kilos. Se les proporcionaba comida cruda que preparaban ellos mismos, mientras que el agua se obtenía de pozos/charcas. Dormían a la intemperie en sacos de dormir y se expusieron a temperaturas que oscilaban entre los 12 y los 42 °C. Datos antropométricos y muestras de sangre en ayunas fueron recogidos al principio y al final del estudio.

Encontramos importantes cambios en la mayoría de los parámetros apuntando hacia valores de antropometría mejorados y función metabólica aumentada. El peso corporal disminuyó una mediana (rango) de –3,8 kg (–12,5 a –0,7), IMC de –1,2 kg/m^2 (–4,4 a –0,2), circunferencia de cadera de –3 cm (–17 a +5), circunferencia de cintura de –5 cm (–18 a +9) e índice cintura/cadera de –0,02 (–0,14 a +0,10). También observamos una disminución (mediana; rango) en: glucosa (–0,6; –1,7 a +0,5 mmol/L), HbA1c (–0,1; –0,4 a +0,2 %), insulina (–4,7; –31,4 a –0,2 pmol/L), HOMA-IR (–1,2; –7,0 a –0,4 mmol*mU/L^2), triglicéridos

(-0,14; -6,12 a +2,18 mmol/L), colesterol total (TC) (-0,7; -2,8 a +0,4 mmol/L), colesterol-LDL (LDL-C) (-0,6; -3,1 a +0,6 mmol/L), índice triglicéridos/colesterol-HDL (HDL-C) (-0,55; -8,98 a 1,34 mol/mol), y FT3 (-0,8; -3,4 a +3,1 pmol/L).

Por otro lado, encontramos que las actividades de ASAT y ALAT aumentaron 11 IU/L (-8 a 54) y 6 IU/L (-13 a 52), respectivamente, mientras que la PCR aumentó 0,56 mg/L (-15,72 a +41,07). El índice ASAT/ALAT aumentó de 0,08 a 1,31 (0,48–2,06).

Nuestras intervenciones podrían estar basadas en causar ´estrés agudo moderado´ adecuado a nuestro estilo de vida moderno, a humanos expuestos a estrés crónico en su vida diaria habitual. El estrés agudo promueve la liberación de hormonas de estrés, incluyendo adrenalina, noradrenalina y cortisol, y cada una de éstas causa profundas adaptaciones metabólicas e inmunológicas. Los factores de estrés agudo aumentan la actividad autonómica, aceleran la proliferación y diferenciación de células inmunes y también estimulan el componente anti-inflamatorio del sistema inmune (i.e. producción de IL-10, lactoferrina, lisozima). De todos modos, el estrés moderado produce inicialmente una respuesta pro-inflamatoria, que puede posteriormente dar lugar a la mejoría del estado reinante de LGI crónica y retorno a la homeostasis.

Un período corto de retorno a las ´condiciones de existencia´ similares a aquéllas en las que se basa nuestro genoma podría mejorar los valores de antropometría y metabolismo mediante un desafío favorable al sistema inmune en sujetos aparentemente sanos y posiblemente en pacientes con fibromialgia. El ´retorno´ podría venir acompañado de algún coste (por ejemplo, infecciones más activas), como compensación de la LGI sistémica crónica típica de nuestro estilo de vida actual de la abundancia. Progresivamente podríamos entender que no podemos tenerlo todo, mientras que las lecciones evolutivas de Darwin y los estudios de intervención nos enseñan que la prevención puede ser más gratificante y asequible que la cultura actual de tratamiento médico.

Ácidos grasos saturados (SFA)

Existe mucha controversia sobre la influencia de las grasas saturadas (SFA) en enfermedad cardiovascular (ECV). La aseveración reinante es que los SFA alimenticios son perjudiciales y que su ingesta, sobre todo la de ácido palmítico (16:0), láurico (12:0) y mirístico (14:0), debería ser limitada o ser ´tan baja como sea posible´. El ácido palmítico se encuentra de manera abundante en, por ejemplo, en grasa animal (leche materna incluida) y aceite de palma, mientras que los ácidos láurico y mirístico son abundantes en el aceite de coco, pero también en la leche materna.

La relación de ácidos grasos saturados con inflamación de bajo grado y enfermedad cardiovascular

El mantra de que la grasa (saturada) alimenticia debe ser minimizada para reducir el riesgo de ECV ha dominado las recomendaciones nutricionales durante décadas. En vista de la controversia actual en relación con su ingesta adecuada y sus efectos, el **Capítulo 2.1** tiene como objetivo resumir la investigación en relación a este heterogéneo grupo de ácidos grasos y los mecanismos que los relacionan con la LGI sistémica (crónica), resistencia a la insulina, síndrome metabólico, y sobre todo, ECV.

El interés en la relación entre grasa alimenticia y ECV se originó a partir de estudios con animales que indicaban que el colesterol alimenticio causaba lesiones arteriales ampliamente mediadas por una elevación de los niveles sanguíneos de colesterol. Desde entonces, la relación entre grasa alimenticia y ECV ha sido estudiada intensamente, usando diferentes abordajes, incluyendo estudios de alimentación controlada, estudios aleatorizados a doble ciego y amplios estudios de cohorte. La mayoría de los estudios sobre SFA se focalizaban exclusivamente en su tendencia a alterar el metabolismo de las lipoproteínas e influir en las concentraciones sanguíneas de las lipoproteínas transportadoras de colesterol, entre otros lípidos. En estos estudios, la pregunta de cuál de todas es la mezcla más sana de las diferentes clases de ácidos grasos se mantiene sin contestar. El indiscutible alto contenido en SFA de la leche materna es un testimonio de sus efectos beneficiosos, al menos en lactantes por, entre otras, sus propiedades antimicrobianas y como fuente de energía rápidamente disponible.

La íntima relación entre inflamación y metabolismo, incluido el metabolismo de glucosa, grasa y colesterol, reveló que la dislipidemia en las sociedades occidentales, sobre todo elevación de triglicéridos, lipoproteínas de baja densidad ´pequeñas-densas´ y lipoproteína de alta densidad ´disfuncional´, está influida por muchos factores de estilo de vida desfavorable. Como ya dijo Paracelso (1493–1541), "Es la dosis (y las circunstancias) las que hacen el veneno". Nuestra comida se compone de sistemas biológicos complejos tales como

carne, pescado, vegetales y frutas, en los cuales los nutrientes, incluidos los SFA, obedecen al equilibrio presente en la materia viva. Es este equilibrio en que los homíninos han evolucionado, el que podría apoyar mejor nuestra salud. Además, es importante obtener una visión de la interacción de los numerosos factores de estilo de vida. El SFA alimenticio es solamente uno de los muchos factores de estilo de vida que juegan un papel en la LGI sistémica crónica y en las subsiguientes adaptaciones metabólicas, incluidas aquellas que originan cambios en las concentraciones de lípidos circulantes y colesterol de lipoproteínas. El medio ambiente nos proporciona muchos otros estímulos pro-inflamatorios además de SFA, pero también muchos estímulos anti-inflamatorios compensadores. Todo es ´equilibrio´.

Afirmamos que es la interacción entre muchos factores en el estilo de vida lo que determina si SFA, y, de hecho, cualquier nutriente, contribuye a LGI sistémica, cambios en el metabolismo de lipoproteínas, y en última instancia, riesgo de ECV. El desequilibrio entre los estímulos pro- y anti-inflamatorios en nuestra sociedad occidental no se origina a partir de una sola causa, y, como consecuencia, puede no ser solucionado por una única ´bala mágica´. La resolución del conflicto entre nuestro medio ambiente artificial, creado por nosotros mismos, y nuestro genoma ancestral podría radicar más en retornar al estilo de vida de la era Paleolítica según la cultura del siglo XXI. Por consiguiente, las recomendaciones nutricionales deberían reconsiderar las directrices de sustituir SFA, ya que ´la comida, y no los nutrientes, es la unidad fundamental en nutrición´. La dieta debería ser investigada en un contexto más amplio, junto a factores no-nutricionales. Esta debería ser una prioridad clara, en contraposición al abordaje reduccionista del estudio de los efectos de nutrientes aislados, como SFA.

Notas añadidas a la revisión de SFA de agosto de 2017

En el **Capítulo 2.1.a**, basado en el reciente ´White paper´ de Pett y colaboradores, nos hemos dado cuenta de que hemos atribuido erróneamente la Figura 1 del **Capítulo 2.1** al Estudio de los Siete Países originado por Ancel Keys. Esta Figura deriva del artículo de Yerushalmy y Hilleboe, publicado un año antes del inicio del Estudio de los Siete Países. Este artículo y sus figuras han sido utilizados para ilustrar los hallazgos del Estudio de los Siete Países en la literatura y en los medios de comunicación social, cuando de hecho, no lo hacen. En base a estos datos, Keys ha sido falsamente acusado de ´elegir datos a la carta´ para hacer ver su punto de vista de la relación entre ingesta de SFA y ECV. Nuestras Figuras 1A y B no fueron producidas en el mismo estudio, y ninguna de ellas era del Estudio de los Siete Países. Dada la inherente naturaleza poco fiable de los datos de comida y mortalidad previos al Estudio de los Siete Países, la pregunta de cuáles fueron los criterios usados por Keys para la selección de aquellos seis países representados en la Figura de 1953 se mantiene.

A pesar de nuestra omisión mencionada anteriormente, no cambiamos nuestra opinión en relación a SFA alimenticio y ECV. El ´White Paper´, de todos modos, no tenía como objetivo este asunto, explicando que "no adopta o promueve ningún consejo nutricional; su intención es únicamente presentar un recuento históricamente preciso de trabajo bien documentado y redirigir las tergiversaciones de ese trabajo". Lo último se refiere al trabajo de Keys como creador del Estudio de los Siete Países.

Los valores en sangre (estado) de ácidos grasos saturados (SFA) y la ingesta de SFA exhiben diferentes relaciones con el colesterol total y las lipoproteínas-colesterol en suero: una explicación de mecanismos centrados en la inflamación de bajo grado inducida por el estilo de vida

El índice colesterol total (TC)/colesterol-HDL (HDL-C) es un factor de riesgo de ECV ampliamente utilizado. En el **Capítulo 2.2** investigamos las relaciones entre los valores de ácidos grasos y los valores séricos de TC, LDL-C, HDL-C e índice TC/HDL-C en cinco grupos étnicos de Tanzania y un grupo holandés, y estudiamos si estas correlaciones eran diferentes de los efectos reportados de la ingesta de SFA en las sociedades occidentales. Los grupos étnicos estudiados difieren ampliamente en las ingestas tanto de SFA como de ácido linoleico (LA). Los valores sanguíneos (estado) de ácidos grasos se determinaron mediante la medición de los ácidos grasos en los ésteres de colesterol en suero y eritrocitos (RBC). Los datos que reflejan la influencia de las ingestas de ácidos grasos en el TC sérico y en el colesterol de lipoproteínas fueron obtenidos de estudios de intervención documentados.

Los habitantes de la isla de Chole tienen altas ingestas de pescado marítimo local y coco, y consumen grandes cantidades de fruta y verdura silvestre. No

usan aceites vegetales para cocinar y presentan ingestas bajas de carbohidratos (CHO) procedentes de cereal o maíz. Las dietas de tanto los Maasai Ruvu como los Wasso se componen de leche cuajada y carne de su propio ganado, a lo que se ha añadido recientemente el *ugali* (gachas de maíz). La ingesta de todo el cuerpo del animal es una práctica común entre los Maasai. El pescado no suele comerse, mientras que la fruta y verdura se consideran comida para las vacas. La gente del Sengerema presenta una ingesta de pescado sistemática (media de 4–5 veces/semana). El *ugali*, *muhogo* (raíz de mandioca) y plátano cocinado son alimentos básicos. Los Hadzabe son cazadores-recolectores tradicionales cuya dieta se compone de bayas, raíces, miel, carne y ocasionalmente, algún pescado. Cazan pequeños animales en la estación húmeda y caza mayor en la estación seca. En los últimos años, el maíz y el aceite de maíz se han convertido de manera inevitable en una gran parte de su dieta contemporánea. La ingesta de grasa en los Países Bajos abarca aproximadamente el 34% de la energía y es, tras los CHO, la mayor fuente de energía. La media de ingestas de SFA, ácidos grasos monoinsaturados (MUFA) y poliinsaturados (PUFA) son de 12,5, 12,7 y 6,3 % de la energía, respectivamente. Los lácteos, carne y productos cárnicos, grasa y bollería son las fuentes principales de grasa alimenticia y SFA en los Países Bajos. Cereales, productos con cereales y bebidas no alcohólicas son las fuentes más importantes de CHO. Más del 75% de la población adulta actual no se ajusta a las recomendaciones actuales para adultos de consumo de 200 g de verdura y 200 g de fruta al día, ni al consumo de una ración de pescado a la semana (particularmente pescado azul). De hecho, el consumo medio de pescado casi ni llega a tres veces al mes, la ingesta de sal se considera demasiado elevada, y también la ingesta de CHO de elevado índice glucémico.

Encontramos que los valores sanguíneos (estado) de 14:0, 16:0 y SFA, pero **no** sus ingestas, se relacionan positivamente con el índice TC/HDL-C. El estado de LA y PUFA y la ingesta de PUFA exhibieron relaciones negativas con el índice TC/HDL-C. Centrándonos en el índice TC/HDL-C el factor de riesgo de ECV usado con frecuencia, encontramos que: 1) basado en el estado de ácidos grasos, el 14:0, 16:0 y SFA tienen efectos perjudiciales, mientas que las ingestas de 14:0, 16:0 y SFA son neutras; 2) tanto el estado como la ingesta de 18:0 son neutros; 3) el estado de MUFA es neutro, mientras que la ingesta de MUFA es beneficiosa; 4) el estado de LA y PUFA son beneficiosos, y lo mismo sucede con la ingesta de PUFA; y por último, 5) el estado de EPA es perjudicial.

Nuestros datos sugieren que, partiendo del índice TC/HDL-C, son los valores elevados en sangre (estado) de SFA, pero **no** una elevada ingesta de SFA, los que se asocian a un aumento de riesgo de ECV, mientras que tanto valores elevados de LA como de PUFA se asocian con un riesgo reducido de ECV. Por tanto, el índice TC/HDL-C es un marcador de riesgo cuestionable, ya que los meta-análisis de ensayos aleatorizados a doble ciego (RCTs) muestran que la sustitución parcial en la dieta de SFA por LA, el PUFA dominante en la alimentación, no afecta al riesgo de ECV. Esta sustitución ha mostrado causar un casi insignificante aumento de la mortalidad por ECV (cociente de riesgo 1,33; 95% de límite de confidencia 0,99–1,79). Concluimos que son muchos los factores del estilo de vida, no solamente la ingesta de SFA, los que determinan el estado de SFA, y sugerimos que es la interacción con muchos otros factores del estilo de vida la que determina si el estado de SFA tiene una contribución relevante a LGI, cambios en lipoproteínas, y riesgo de ECV. Estos resultados pueden enseñarnos a considerar los efectos sobre la salud de toda la dieta junto con muchos otros factores de estilo de vida no alimenticios, en contraposición al abordaje reduccionista de estudiar los efectos de nutrientes aislados, incluyendo a SFA y PUFA.

Comentario sobre el informe ´Dietary Fats and Cardiovascular Disease: A Presidential Advisory from the American Heart Association (AHA)´

Recientemente, la Asociación Americana del Corazón (AHA) ha publicado un meta-análisis haciendo énfasis en su recomendación previa de limitar la ingesta de SFA. SFA debería ser sustituida por grasa insaturada, sobre todo PUFA, para reducir la incidencia de enfermedad cardíaca. Reivindican que este intercambio reduce el riesgo de episodios cardiovasculares aproximadamente en un 30%; una reducción del riesgo comparable al tratamiento con estatinas. La AHA también está en contra del consumo de aceite de coco porque aumenta el LDL-C y ´no tiene efectos compensatorios favorables´. Argumentamos que la concentración de LDL-C es un criterio de evaluación subjetivo, y no una patología, mientras que no hay estudios que muestren efectos no favorables del aceite de coco sobre criterios de evaluación objetivos. La AHA realiza importantes exclusiones de estudios en

sus meta-análisis, pero no aplica criterios estrictos en la elección de los cuatro ensayos que constituyen la columna vertebral de su meta-análisis final. Uno de éstos no era un ensayo a doble ciego aleatorizado, mientras que otro presentaba un ´sesgo de procedimiento´. El estudio negativo de mayor tamaño fue excluido, entre otras razones, porque no tuvo una duración mínima de dos años. El meta-análisis de la AHA cumple con el concepto de ´elegir datos a la carta´. Existen actualmente al menos nueve revisiones de expertos que no han conseguido encontrar una relación clara entre SFA, mortalidad cardiovascular y mortalidad total. Argumentamos que los individuos con síndrome metabólico deberían tener cuidado con SFA y CHO en la alimentación, ya que sintetizan SFA *de novo* a partir de CHO y ahorran los SFA procedentes de la alimentación. El riesgo elevado de los individuos con síndrome metabólico no es razón para limitar el consumo de SFA en la población genuinamente sana. Algunos SFA son definitivamente pro-inflamatorios, pero una alimentación equilibrada también contiene componentes anti-inflamatorios.

Astaxantina, el carotenoide rosa

El **Capítulo 3** se centra en la astaxantina. La astaxantina es un carotenoide único, predominantemente de origen marino. La forma natural actúa como un antioxidante sin propiedades antioxidantes o efectos secundarios tras la ingesta oral. La astaxantina pertenece a la familia de las xantofilas, proporcionando el color rojo-rosado a algunas microalgas (i.e. *Haematococcus pluvialis*) y se acumula en varios animales de posición más elevada en la cadena alimenticia, como flamencos, salmón, camarones y cigalas. Es probable que la astaxantina forme parte de ecosistema tierra-agua del que procede el *Homo sapiens*. La molécula se extiende de lado a lado de la bicapa de fosfolípidos de las membranas celulares debido a sus dos cabezas polares que están separadas por una cadena de carbonos ramificada con nueve enlaces dobles conjugados. La astaxantina puede aumentar la respuesta inmune, disminuir los síntomas relacionados con el daño por estrés oxidativo, y se ha probado efectiva en muchas enfermedades y afecciones, como enfermedad de Alzheimer, obesidad, asma, próstata agrandada, artrosis y artritis reumatoide.

Cinética de astaxantina en plasma y eritrocitos en sujetos sanos tras una única dosis oral y una dosis oral de mantenimiento

El **Capítulo 3.1** presenta la cinética de la astaxantina en sujetos sanos. Tras su ingesta, la astaxantina ha demostrado alcanzar un pico en plasma tras aproximadamente 7 h y disminuir su concentración con una vida mediana de aproximadamente 21 h. Investigamos la cinética de astaxantina en plasma y glóbulos rojos (RBC) en cuatro adultos sanos tras la toma de una única dosis de 40 mg. La astaxantina en plasma y RBC fue medida durante 72 h. A continuación, se administró una dosis de 8 mg/día durante 17 días. Cada mañana se midió la astaxantina en plasma y RBC.

La astaxantina en plasma alcanzó un pico (de 79 a 315 nmol/L) tras 8 horas y tras ello bajó (vida media 18 h). En 72 h, la astaxantina en plasma había vuelto a la concentración de base. La astaxantina en RBC alcanzó un pico (de 63 a 137 nmol/L de concentrado de RBC) a las 12 h, y posteriormente desapareció (vida media, 28 h). A lo largo de la dosis diaria de 17 días, la astaxantina aumentó hasta el día 10 (187 nmol/L) y después disminuyó a una concentración estable similar a la alcanzada tras dos días. La concentración de astaxantina en RBC parece ser altamente variable (concentración mediana del grupo, 86 nmol/L de concentrado de RBC).

Encontramos elevadas variaciones intra- e inter-individuales, sobre todo en RBC, posiblemente debido a una falta de estandarización en la diferencia de tiempo entre la ingesta de astaxantina y la toma de muestras, a fluctuaciones en el contexto alimenticio de la ingesta, biodisponibilidad variable, amplio volumen de distribución, degradación u otros. La astaxantina oral se absorbe y se incorpora rápidamente a los RBC. Las cortas vidas medias de la astaxantina en plasma y RBC de 18 y 28 h, respectivamente, sugieren la necesidad de tomar astaxantina de manera diaria para mantener un estado estable mayor que el de base, al menos en la fase inicial de suplementación, cuando el equilibrio total del cuerpo, si existiera, no se ha alcanzado aún. Esta fase temprana podría estar influida en parte por la tendencia de la astaxantina a incorporarse a todas las membranas celulares corporales.

La suplementación de astaxantina a pacientes con enfermedad de células falciformes aumenta la astaxantina en plasma y eritrocitos y podría mejorar el componente hemolítico de la enfermedad

La enfermedad de células falciformes (SCD) es un desorden heterogéneo que se caracteriza en sus mecanismos de acción por un componente hemolítico y uno vaso-oclusivo. Este último da lugar a daño isquémico en órganos acumulativo que puede ocasionalmente precipitar dolorosas crisis vaso-oclusivas, y todo en conjunto contribuye a una calidad de vida disminuida y muerte temprana. El componente hemolítico podría tener un desencadenante importante en la generación por parte de la hemoglobina falciforme (HbS) de especies de oxígeno reactivas cerca de la membrana de RBC sensible a peroxidación de lípidos, culminando en hemolisis si el desafío no es contrarrestado adecuadamente. El componente vaso-oclusivo podría estar ampliamente originado por el anteriormente mencionado componente hemolítico.

Tratar el componente hemolítico, y sobre todo el estrés oxidativo, mediante la mejora del devastador componente vaso-oclusivo, parece una estrategia de intervención lógica. Se han publicado varios estudios con antioxidantes naturales con resultados prometedores, incluyendo algunos con vitamina E, curcuminoides, extracto de ajo envejecido, N-acetil-cisteína y zinc. Debido a sus propiedades antioxidantes únicas, la astaxantina podría mejorar el componente hemolítico de SCD.

El **Capítulo 3.2** presenta un estudio sin enmascaramiento, donde incluimos 10 pacientes ambulantes con SCD confirmada por examen de laboratorio (siete adultos, tres niños, de los cuales tres hombres y siete mujeres, edad media 31 años, rango 6–52 años) del Centro Médico de Sint Maarten. Investigamos el efecto de una dosis oral diaria de 8–12 mg durante tres meses en los niveles de astaxantina en plasma y RBC (objetivo primario) y varios parámetros hematológicos y clínicos (objetivo secundario), incluyendo número de reticulocitos, volumen corpuscular medio (MCV), amplitud de distribución de RBC (RDW), lactato deshidrogenasa (LDH) y dimetilarginina asimétrica (ADMA).

El valor base de astaxantina en plasma (33 nmol/L) y RBC (11 nmol/L concentrado de RBC) aumentó hasta 225, 174, 167 nmol/L (plasma) y 149, 100, 71 nmol/L concentrado de RBC tras 1, 2 y 3 meses, respectivamente. Los reticulocitos disminuyeron desde el inicio y 2 meses (9,5 y 8,8%) hasta los 3 meses (5,6%), MCV de 2 a 3 meses (88 a 86 fL), MCH desde el inicio hasta los 3 meses (30 a 28 pg) y RDW desde el inicio y 2 meses (19,2 y 19,0%) hasta los 3 meses (16,7%). La arginina plasmática disminuyó de los 2 a los 3 meses (46,6 a 39,4 µmol/L). La suplementación con astaxantina no produjo cambios en homoarginina, ADMA, dimetilarginina simétrica (SDMA) ni en el índice ADMA/arginina. Los reticulocitos al inicio se correlacionaron con los cambios relativos en reticulocitos desde el inicio a los 3 meses. Los cambios relativos en reticulocitos se correlacionaron con los cambios relativos de RBC, RDW, LDH, ALAT, pero no con los cambios en hematocrito dentro del mismo período.

Concluimos que la astaxantina se incorpora en los RBC de pacientes con SCD y podría afectar favorablemente al componente hemolítico. Encontramos una ligera reducción del número de reticulocitos tras 3 meses, probablemente indicando una menor hemólisis, mientras que muchos pacientes de este estudio sin placebo-control y sin aleatorizar, comentaron que se ´sentían mejor´. Se recomienda un estudio aleatorizado a doble ciego más grande, usando una dosis similar o mayor, preferiblemente durante más de 3 meses. Podría ser incluso mejor incluir astaxantina en un mix de suplementos con otros antioxidantes (por ejemplo, baja dosis de vitamina E, betacaroteno, vitamina C y ácido folico), minerales (selenio si fuera necesario; y, sobre todo, zinc); aminoácidos (sobre todo arginina), aceite de pescado y vitamina D. Los antioxidantes no trabajan de manera aislada, sino que son parte de una red aun pobremente entendida de antioxidantes, de neutralizadores de radicales libres, queladores y enzimas antioxidantes, y, por tanto, parece improbable encontrar una única ´bala mágica´ para prevenir o tratar cualquier enfermedad asociada con estrés oxidativo.

Mayor prevalencia de 'síndrome de T3 baja' en pacientes con fatiga crónica: un estudio caso-control

El síndrome de fatiga crónica (CFS) es una enfermedad heterogénea de causa(s) desconocida. Se han hipotetizado muchas cascadas fisiopatológicas, pero raramente se encuentran situaciones orgánicas subyacentes. En CFS se ha encontrado un eje hipotálamo-hipófisis-adrenales (HPA) alterado, presentado como hipocortisolismo moderado, *feedback* negativo aumentado y respuestas disminuidas a cualquier desafío. Los síntomas de CFS se parecen a un estado de hipotiroidismo, posiblemente secundario a una inflamación crónica (de bajo grado) (metabólica). Podríamos estar tratando con el equivalente humano a la ´hibernación´ con causas que se originarían en un

estilo de vida típico occidental y que desencadena inflamación de bajo grado (LGI).

En el **Capítulo 4** presentamos un estudio caso-control con CFS. Investigamos a 98 pacientes con CFS (21–69 años, 21 hombres) y 99 controles de la misma edad y sexo (19–65 años, 23 hombres). Medimos parámetros de función tiroidea, inflamación (metabólica), integridad de la pared intestinal y nutrientes que influyen en la función tiroidea y/o inflamación.

Lo más notable fue que los pacientes de CFS mostraron una TSH similar, pero menor T3 libre (FT3) (diferencia de medianas 0,1%), T4 total (TT4) (11,9%), T3 total (TT3) (12,5%) y % de TT3 (4,7%), mayor % de T3 reversa (rT3) (13,3%), y menor excreción de yodo en la orina de 24-h (27,6%). La FT3 por debajo de los valores de referencia, consistente con el ´síndrome de T3 baja´, se encontró en 16/98 pacientes con CFS vs. 7/99 controles (OR 2,56; 95% CI=1,00–6,54). La mayor parte de las observaciones se mantuvieron en dos análisis de sensibilidad con valores de corte más estrictos para índice de masa corporal, PCR ultra sensible (hs-CRP) y leucocitos. Encontramos una evidencia posible de inflamación (crónica) metabólica de bajo grado (ferritina y HDL-colesterol). FT3, TT3, TT4 y rT3 se correlacionaron positivamente con hsCRP en pacientes con CFS y en todos los sujetos. Sólo TT3 y TT4 se correlacionaron positivamente con hsCRP en controles.

Los bajos niveles circulantes de T3 y el cambio aparente de T3 a rT3 podrían reflejar niveles de T3 severamente deprimidos en los tejidos. Los hallazgos presentes podrían estar en línea con estudios metabolómicos recientes que señalan a un estado hipometabólico. Se asemejan a una forma leve de ´síndrome de enfermedad no tiroidea´ y al ´síndrome de T3 baja´ experimentado por un subgrupo de pacientes hipotiroideos recibiendo monoterapia con T4. Nuestro estudio necesita confirmación y extensión por otros. Si se confirmara, ensayos con, por ejemplo, suplementos de T3 y yodo podrían estar indicados. Parece ser que el CFS es una enfermedad heterogénea con un camino fisiopatológico común. Los hallazgos actuales están posiblemente en línea con camino final común, pero no nos llevan más cerca de la(s) causa.

Epílogo. Aplicabilidad de un abordaje evolutivo a las enfermedades (crónicas) y su prevención

La ´Medicina Evolutiva´ está ganando aceptación. La influyente ´The Lancet´ ha publicado recientemente tres artículos y una editorial sobre el tema [1-4]. Algo lógico, pero ahora ya demostrado científicamente, es que el Estilo de Vida, y no la Genética, es la única y más importante causa de las enfermedades ´típicamente occidentales´ de la abundancia [5].

En esta tesis esperamos haber contribuido al concepto mencionado anteriormente, y más específicamente, a la conciencia de que el ´envejecimiento saludable´ es una cuestión de estilo de vida. Los componentes de un estilo de vida insalubre son una dieta desequilibrada, actividad física insuficiente; sueño inadecuado, estrés crónico, flora microbiana anormal (microbioma) y ambiente nocivo (por ejemplo, fumar, polvo fino). Debido a su interacción, podría no ser tan productivo el estudiar estos factores de riesgo de manera aislada, sea por medio de estudios aleatorizados a doble ciego (RCTs) o no.

Ha habido, y sigue habiendo, poca atención a las limitaciones de los RCTs y sobre todo, a las limitaciones de los meta-análisis sobre ellos. Rawlins [6] apuntó que: "las jerarquías de la evidencia deberían ser reemplazadas por la aceptación—y sobre todo adopción—de una diversidad de abordajes." De todos modos, los RCTs y sus meta-análisis son extensamente vistos como el ´nivel más elevado de evidencia´, y por algunos incluso como ´los únicos a tener en cuenta para realizar recomendaciones´. Este abordaje, más apropiado para fármacos, pero no necesariamente para nutrientes, ha alcanzado el estado actual de ´paradigma´, definido como ´un entendimiento compartido por los científicos o alumnos que trabajan en una disciplina, sobre problemas, estructuras, valores y asunciones importantes que determinan dicha disciplina´ [7].

Desde el principio, los RCTs y sus meta-análisis no estaban destinados a constituir los únicos componentes de la ´Medicina/Nutrición Basada en Evidencia´, al menos no en las mentes de los inventores originales [8, 9]. Además, los meta-análisis actuales llegan a menudo a conclusiones opuestas, incluso tras leer exactamente la misma literatura. Un ejemplo es la reciente recomendación de la AHA sobre grasa saturada basada en meta-análisis, que contradice "un total de al menos 17 meta-análisis y revisiones sistemáticas y cinco revisiones no sistemáticas que no consiguieron encontrar una conexión clara entre grasas saturadas y enfermedad coronaria/muerte cardiovascular" [10]. La omisión selectiva de estudios debido a ´calidad pobre´, inspirada posiblemente en opinión sesgada, también denominada ´elección a la carta´, podría

haberse convertido en la nueva tendencia, y está, al menos, en el punto de mira hoy en día [11–13].

Tales controversias no otorgan mucha confianza a la ciencia, y frustran al público. Hoy en día, todo el mundo tiene virtualmente acceso ilimitado a la literatura científica, y cualquier ´opinión científica´ no debería darse por sentada, aunque provenga de, i.e. un eminente científico (también denominado ´Medicina Basada en la Eminencia´), Consejo de Salud, o Gobierno.

Lo que hoy en día entiende (la mayoría de) el público y (la mayoría de) los Gobiernos es el concepto de ´sostenibilidad´, definido en ecología como ´la propiedad de los sistemas biológicos de mantenerse con diversidad y productivos indefinidamente´ [14]. Ahora que las consecuencias de, por ejemplo, el calentamiento global, están empezando a manifestarse, están surgiendo cada vez más preguntas incisivas como ´qué hay en mi comida´, ´cómo se está produciendo´ y ´por qué mi abuela no reconoce esto como comestible´?

El público entiende, posiblemente mejor que muchos científicos, que hemos creado un conflicto entre nuestro medio ambiente y nuestro genoma ancestral. Este último no puede adaptarse, ni genéticamente (largo plazo), ni epigenéticamente (menor plazo), a los desafíos medioambientales que suceden cada vez de una manera más rápida. La controversia se debe, no solamente a los frecuentemente inculpados ´bloggers´ [15–18], y no es solamente sobre grasas saturadas (como uno de los temas en esta tesis), pero también carbohidratos, ácido linoleico, sal y vitamina D, entre otros.

En vista de todo lo anterior, concluimos que la única manera efectiva de llegar a un ´envejecimiento saludable´ podría ser volver al estilo de vida de la era Paleolítica, según la cultura del siglo XXI. Y de eso exactamente versa la ´Medicina Evolutiva´.

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Curriculum Vitae

Begoña Ruiz Núñez was born on November 11, 1978 in Bilbao, Spain. She graduated *cum laude* at IB La Serna (Madrid) and she completed the degree in Physiotherapy at the Universidad Complutense de Madrid, also *cum laude*. After following several postgraduate courses to improve her education while working as a physiotherapist and osteopath for more than 10 years, she came across clinical Psycho-Neuro-Immunology (cPNI), a discipline that approaches patient care from an evolutionary point of view. From 2005 on, she combined patient care with teaching cPNI, physiotherapy and osteopathy. In the meanwhile, she opened her own clinic in Madrid and in 2008 she began a Master of Science (MSc) in cPNI at the Universidad de Girona, Spain, which allowed her to start her PhD in 2010.

During her PhD project, described in this thesis, she moved to Groningen and left patient care during the five years she spent in The Netherlands, where she worked under the supervision of Profs. F.A.J. Muskiet, I.P. Kema and Dr. D.A.J. Dijck-Brouwer. Since 2015, under the name of *Healthy Institute*, her own brand; she teaches evolutionary medicine and cPNI to professionals in patient care. Since 2011 she collaborates as a scientific advisor for a supplement brand and gives divulging talks about evolutionary medicine to the public and therapists. She is also the President of the Asociación Española de Psiconeuroinmunología clínica (AEPNIc) and works as therapist from an evolutionary perspective, guiding them through lifestyle changes in order to achieve health.

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